Applied Energy 179 (2016) 124–135

Contents lists available at ScienceDirect

Applied Energy

journal homepage: www.elsevier.com/locate/apenergy

Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance

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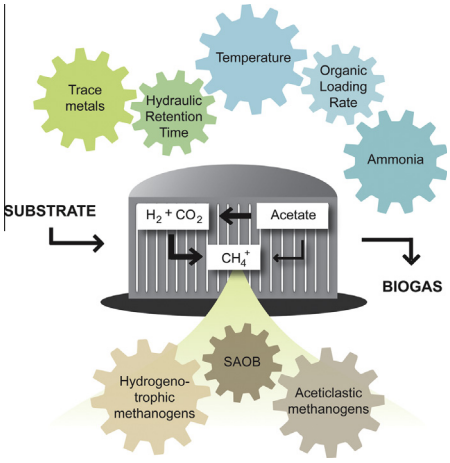
HIGHLIGHTS

- Syntrophic acetate oxidation (SAO) dominates in ammonia-adapted biogas processes.
- SAO bacteria compete for acetate and depend on their methanogenic partner.
- Syntrophic acetate oxidisers are present under a wide range of operating conditions.
- Ammonia, acetate, temperature, retention time and trace elements influence SAO.
- Awareness of SAO enables strategies for process optimisation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 1 June 2016
Accepted 17 June 2016

Keywords:
Syntrophic acetate oxidising bacteria
Methanogens
Ammonia inhibition
Operational parameters
Trace metals
Temperature

ABSTRACT

Anaerobic degradation of protein-rich materials has high methane potential and produces nutrient-rich residue, but requires strategies to avoid ammonia inhibition. A well-adapted process can cope with substantially higher ammonia levels than an unadapted process and analyses of pathways for methanisation of acetate, combined with determination of microbial community structure, strongly indicate that this is due to a significant contribution of syntrophic acetate oxidation. The microorganisms involved in syntrophic acetate oxidation thus most likely occupy a unique niche and play an important role in methane formation. This review summarises current insight of syntrophic acetate oxidising microorganisms, their presence and the detection of novel species and relate these observations with operating conditions of the biogas processes in order to explore contributing factors for development of an ammonia-tolerant microbial community that efficiently degrades acetate through the syntrophic pathway. Besides high ammonia level, acetate concentration, temperature and methanogenic community structure are considered in this review as likely factors that shape and influence SAO-mediated microbial ecosystems. The

Abbreviations: VFA, volatile fatty acids; SAO, syntrophic acetate oxidation; SAOB, syntrophic acetate-oxidising bacteria; HRT, hydraulic retention time; qPCR, quantitative PCR; FTHFS, formyl tetrahydrofolate synthetase; DNA-SIP, nucleic acid-based stable carbon isotopic probing; MAR-FISH, microautoradiography-fluorescence in situ hybridisation; TAN, total ammoniacal nitrogen; OLR, organic loading rate; VS, volatile solid; COD, chemical oxygen demand; RT-PCR, reverse transcription PCR; *mcrA*, methyl coenzyme-M reductase; T-RFLP, terminal restriction fragment length polymorphism; NanoSIMS, nanometer scale secondary-ion mass spectrometry; ARISA, automated ribosomal intergenic spacer analysis; DIET, direct interspecies electron transfer; UASB, upflow anaerobic sludge blanket.

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http://dx.doi.org/10.1016/j.apenergy.2016.06.061
0306-2619/

main purpose of this review is to facilitate process optimisation through considering the activity and growth of this key microbial community.

Contents

1. Introduction

Along with increased energy efficiency, substitution of fossil fuel-derived energy with renewable sources is crucial in achieving the goal of reduced emissions of anthropogenic greenhouse gases. Biogas produced through anaerobic degradation of organic residues has good potential in climate change mitigation and also involves indirect environmental benefits such as reduced emissions of air pollutants and ammonia. European biogas production is experiencing high growth at the moment [1], increasing the demand for establishment of new production plants, but also process optimisation to increase energy output in existing plants.

Protein-rich substrates are of interest for commercial biogas production due to the relatively high methane yield potential [2,3] and the high level of plant-available ammonium (NH_4^+-N) in the residue. This residue can be applied to arable land as fertiliser, which reduces the need for production of mineral fertiliser, contributes to recirculation of nutrients and improves soil quality. A high content of ammonium considerably increases the value of the residue and thereby enhances profits for the biogas plant. However, due to the high amounts of ammonium in equilibrium with ammonia, the anaerobic degradation of protein-rich substrates is often associated with process instability, indicated by reduced biogas production and/or methane content, fluctuations in pH and alkalinity, and accumulation of volatile fatty acids (VFA) [4]. Protein-rich substrates are also a common source for formation of sulphide [5,6], which is not only toxic for various microbial populations but also forms complexes with metals, resulting in decreased bioavailability of trace elements essential for microbial activity [7]. However, the positive factors are still strong incentives for commercial biogas plants to operate at high ammonia, resulting in demands for solutions and strategies to handle the associated problems.

Suggested physical and chemical solutions to handle the complications associated with nitrogen-rich material include dilution of substrate, air-stripping, ammonia recovery through integration of a microbial desalination cell and inclusion of material with ion exchange capacity or carbon fibre [3,4,8,9]. Furthermore, the importance of microbial adaptation to high ammonia levels has long been emphasised in the literature [4], indicating the necessity for allowing the microbial community to acclimatise to the prevailing conditions for successful operation. Recent achievements in analyses of pathways for methanisation of acetate, combined with determination of microbial community structure, provide strong indications of a significant contribution of syntrophic acetate oxidation (SAO) to methane formation in high-ammonia processes [2,10–16]. Consequently, operating parameters enhancing the activity and/or growth of key microbial constituents could potentially result in significantly improved process stability and biogas yield. Hence, this review sought to correlate current insights into microbial structures and dynamics, growth conditions of the microorganisms involved and the influence of operating parameters in SAO-mediated processes.

2. Ammonia inhibition

The dominant influence on ammonium-nitrogen concentration in digester sludge is the nitrogen content of the substrate. Organic waste streams originating from animal breeding (slaughterhouse waste, dairy wastewater stream, animal manure, aquaculture sludge) and ethanol fermentation (distiller's waste) are examples of ammonia- and protein-rich substrates commonly used for current biogas production [3–5,17,18]. The nitrogen level in certain food industry and household wastes can also be enough to perturb digester operation [19]. In addition, the level of ammonium-nitrogen is dependent on the degree of decomposition of the process, i.e. the proportion of the organic material converted to methane. A smaller

proportion of the organic nitrogen in the substrate is mineralised to ammonium-nitrogen at low compared with higher degree of decomposition, which in turn is dependent on sludge retention time, temperature and the microbial community [20]. Moreover, temperature and pH indirectly affect the level of inhibition, since these parameters regulate the equilibrium between ammonium (NH_4^+) and ammonia (NH_3) in the sludge. As shown in Eq. (1), increased temperature and pH shift the ratio towards NH_3, which is reported to be the actual cause of microbial inhibition [21].

$$NH_3\text{-}N = \frac{NH_4^+\text{-}N}{1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T}\right)}}}\ [21] \quad (1)$$

In this equation NH_4^+-N is the total ammonia-nitrogen ($NH_4^+ + NH_3$) and T is the temperature (kelvin). The impact of increased temperature is further enhanced by the reduced solubility of carbon dioxide, which increases the pH and thereby shifts the equilibrium further towards the toxic ammonia.

Nevertheless, the actual digester response to ammonium depends on the microbial community, which in turn is influenced by inoculum, substrate characteristics and operating parameters. Total and free ammonia concentration, together with temperature, have been identified as the main influencing factors determining bacterial community structure in full-scale anaerobic digesters [22]. The inhibitory effects of ammonia on the microbial consortia are also considered to have a pronounced impact in later stages of degradation, involving the activity of hydrogen/formate-utilising (hydrogenotrophic) or acetate-utilising (acetoclastic) methanogens, where the acetoclastic methanogens (*Methanosaeta* sp. and certain *Methanosarcina* sp.) are considered to be most sensitive to ammonia [4]. Since biogas production through anaerobic degradation of organic components demands complex microbial communities, with close interspecies cooperation, the reduced methanogenic activity subsequently influences reaction pathways higher up in the degradation chain [23].

3. Syntrophic acetate oxidation

Conditions resulting in inhibition of acetate-utilising methanogenic communities, such as high concentrations of ammonia, are believed to result in appearance of microbial competitors for acetate and in numerous studies have been suggested to promote the development of SAO [2,6,10,12,14–16,24–38]. The initial step of the sequential SAO reaction involves oxidation of acetate to hydrogen and carbon dioxide and formate by syntrophic acetate-oxidising bacteria (SAOB), followed by consumption of these products by hydrogenotrophic methanogens for the generation of methane.

3.1. Syntrophic acetate-oxidising bacteria

The currently known bacteria capable of syntrophic acetate oxidation are the thermophilic *Thermacetogenium phaeum* [39] and *Pseudothermotoga lettingae* [40,41], the thermotolerant *Tepidanaerobacter acetatoxydans* [42] and the mesophilic *Clostridium ultunense* [43] and *Syntrophaceticus schinkii* [44]. Syntrophic acetate oxidisers are considered slow growers [45], which can be a disadvantage in the competition for acetate with the acetoclastic methanogens. Nevertheless, hydrogenotrophic methanogens [46] and a majority of the SAOB possess relatively high ammonia tolerance [42–44,47,48], a feature that probably provides them with a competitive advantage in ammonia-stressed systems.

3.1.1. Presence and relative abundance in anaerobic digestion processes

Genes affiliated to known SAOB have been detected in digesters operating under diverse conditions in terms of ammonia concentration, temperature, hydraulic retention time (HRT), substrate feed and digester configuration [11–13,16,17,29,49–61]. On correlating the SAOB gene abundance obtained in the different anaerobic digesters, the levels appear to be interrelated depending on the prevalent acetate degradation pathway. In mesophilic, high-ammonia digesters with syntrophic acetate oxidation as the key pathway, species-specific quantitative PCR (qPCR) analyses have determined the abundances of genes affiliated to *C. ultunense*, *S. schinkii* and *T. acetatoxydans* to range between 10^{6-11}, 10^{6-9} and 10^{4-10} per mL, respectively. The corresponding levels in digesters operating at low ammonia and dominated by the acetoclastic pathway are 10^{5-7} for *S. schinkii*, whereas *C. ultunense* and *T. acetatoxydans* levels vary from below threshold up to 10^5 and 10^3, respectively [6,11–13,16,57]. However, it is noteworthy that the levels of these syntrophic bacteria are relatively low in comparison with the total bacterial gene abundance, despite confirmation of SAO-mediated acetate degradation. In mesophilic digesters, 0–0.04%, 0–7.0% and 0–3.0% of total bacteria community has been shown to be represented by *C. ultunense*, *S. schinkii* and *T. acetatoxydans*, respectively [11,13,16]. In thermophilic digesters, both similar (0–0.7%) [13] and higher relative levels [52] have been

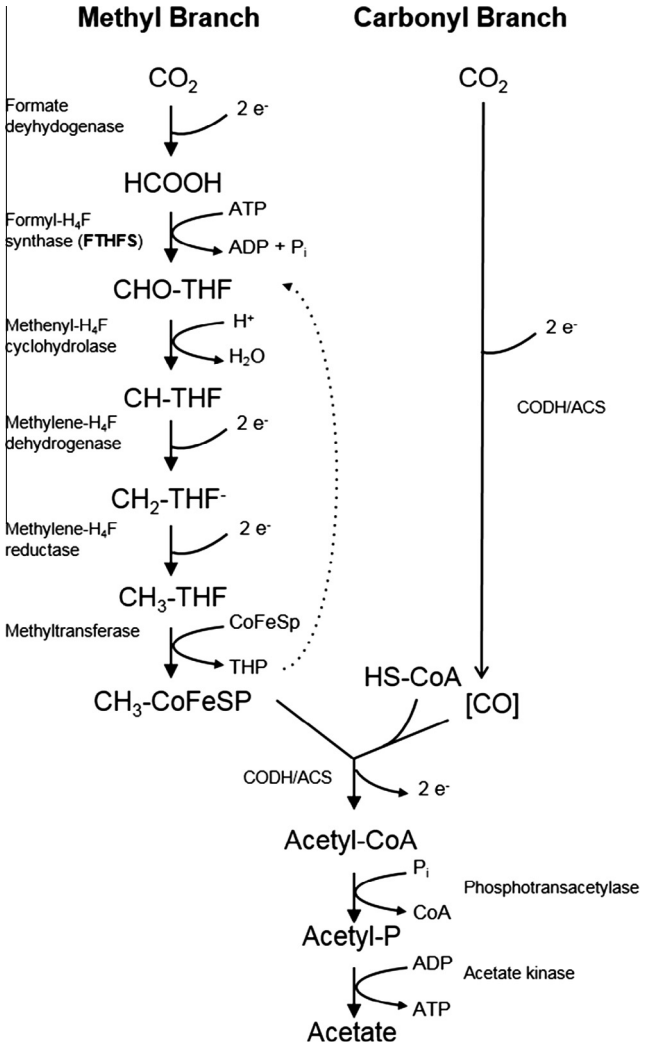

Fig. 1. The Wood–Ljungdahl pathway (also called the Acetyl-CoA pathway). The pathway comprises two branches, the methyl and the carbonyl branch, and involves a series of reactions resulting in the reduction of two carbon dioxide molecules and the final production of acetate. During the process no net ATP is formed and energy production is dependent on chemiosmotic processes coupled to the translocation of protons or sodium ions [64].

reported. In the study with higher relative levels, 0.8–19% of the total analysed sequences were allocated to the genome of a defined syntrophic acetate oxidiser (based on 97% maximum identity) [52].

3.1.2. Detection of novel syntrophic acetate-oxidising bacteria

Distant relatedness based on the 16S ribosomal RNA gene and the strict requirements of current cultivation techniques have constrained the detection and characterisation of novel SAOB. Approaches based on targeting functional genes hold promise for identification of key players in SAO and assessment of the link between function and microbiology. The majority of the characterised SAOB have been positioned in the physiological group of homoacetogens, which is defined by use of the Wood-Ljungdahl pathway during growth on autotrophic and/or heterotrophic substrates and production of mainly acetate [62,63]. In this acetogenic pathway, formyl tetrahydrofolate synthetase (FTHFS) is a key enzyme catalysing the ATP-dependent activation of formate (Fig. 1).

For syntrophic functioning, SAOB have been postulated to reverse the Wood-Ljungdahl pathway for oxidation of acetate [62,63]. However, the SAOB *P. lettingae* does not use the Wood-Ljungdahl pathway [45] and recently an additional pathway for acetate oxidation, combining the methyl branch of the Wood-Ljungdahl pathway with a glycine cleavage system, have been suggested [65]. However, irrespective of pathway, all SAOB have been shown to access at least one and sometimes two FTHFS genes, implying that *fhs* (gene encoding FTHFS) profiling is suitable for delineation of populations expressing SAOB capabilities. Profiling of acetogenic communities in anaerobic digesters has revealed shifts in the acetogenic community, concurrently with ammonia-induced introduction of SAO in mesophilic anaerobic digesters [66] and has identified potential acetate-oxidising syntrophs in thermophilic SAO cultures [67]. Recently designed primers, expanding the recovery of *fhs* genes and including targeting of known syntrophic acetate oxidisers [63,68], retrieved *fhs* gene sequences from various biogas digesters that formed distinct phylogenetic clusters. Only a few genotypes were shared with previous findings in other anaerobic environments (e.g. rumen, termite gut, horse manure) [16,68,69]. Correlation between high abundance of *fhs* genes with members of the order *Clostridiales* and *Thermoanaerobacterales* and ammonia-induced SAO dominance further reinforced the importance of the acetogenic bacteria represented and promotes their position as potential acetate-oxidising syntrophs [68]. Through a metagenome sequencing approach and identification of genes encoding enzymes involved in the Wood-Ljungdahl pathway, Zakrzewski and co-authors [70] suggested a syntrophic association between an unknown *Thermacetogenium* species and *Methanothermobacter thermautotrophicus*. Sequences assigned to the phylum *Thermotogae* and the genus *Clostridium* were also found in the study and suggested to be involved in SAO. In another recent study, genes encoding enzymes involved in the Wood-Ljungdahl pathway were analysed in mesophilic digesters with gradually increased contribution of SAO from 5% to 25% [15]. Nucleic acid-based stable carbon isotopic probing (DNA-SIP) of enrichment cultures from the digesters was another approach used in that study to reveal distinctive bacterial communities for this syntrophic function. However, appearance of keystone bacterial populations responsible for SAO was not detected, leading the authors to question whether syntrophic acetate oxidation is the work of a defined species and suggest that a diverse array of bacterial taxa within dynamic heterogeneous communities may instead be responsible for the syntrophic reaction.

Acetate utilisation by *Synergistes* group 4 has been demonstrated by the microautoradiography-fluorescence in situ hybridisation (MAR-FISH) technique with ^{14}C-acetate. This *Synergistes* group was suggested to mediate acetate conversion through SAO and was shown to have competitive advantages over *Methanosaeta* at high acetate concentrations (2.5–10 mM) [71]. By incubating a sample from a full-scale biogas plant with [U-^{13}C]acetate, followed by mapping of expressed and labelled proteins onto a binned metagenome Mosbaek et al. [72] recently suggested that *Methanosarcina*, *Methanoculleus* and five subspecies of *Clostridia* were actively involved in SAO. Other studies have proposed possible new SAOB due to changed abundances in response to altered environmental conditions in enrichment cultivations. Yamada and co-authors [73] observed dominance of bacteria with high identity to *Tepidanaerobacter syntrophicus* and *Coprothermobacter proteolyticus* in thermophilic acetate enrichments. The cultures had been supplemented with conductive iron oxide particles with the prospect of facilitating electric syntrophy in the methanogenesis of acetate. In another study, stable carbon isotopic analysis combined with pyrosequencing indicated that *Coprothermobacter* spp. was the main acetate degrader in syntrophic association with hydrogenotrophic *Methanothermobacter* in a high-rate (HRT of 2–4 days) and high temperature (65 °C) anaerobic digester [74]. Increased abundance of *Spirochaetes* has been observed in acetate-fed batch cultivations and it is suggested as a potential SAOB candidate [75]. Bassani and co-authors [76] recently conducted a study of a two-stage system where external H_2 was added to the second digester in order to increase the proportion of methane in the biogas. An increase in hydrogenotrophic methanogens within the *Methanoculleus* genus was noted in the hydrogen-supplemented digester, concurrently with a rise in *Thermoanaerobacteraceae*, which were therefore proposed to be involved in SAO.

To conclude, the limited number of isolates characterised restricts research about syntrophic communities and their interaction with the ambient microbial society and responses to environmental parameters. In addition to further isolations, a prerequisite for increased knowledge is the detection of novel SAOB in the complex digester environment. We believe that approaches targeting the *fhs* gene or DNA-SIP are promising techniques in this regard.

4. Influence of digester conditions and operating parameters on SAO

4.1. Ammonia threshold and microbial adaptation

Ammonia-induced perturbation of anaerobic laboratory-scale digesters often appears concurrently with increased loading of ammonia-rich organic substrate, which complicates the distinction between overload and ammonia as the cause of digester upset. However, disturbance of anaerobic degradation at equivalent ammonia levels, irrespective of the organic loading rate, was recently demonstrated [69]. Thus, here we consider the ammonia level as the main cause of digester instability, neglecting the impact of disparity of applied organic loads between the studies reviewed.

Digester disturbance, reflected by increased VFA concentrations and reduced gas production rate and methane yield, has been observed at levels exceeding 0.08–0.2 g NH_3-N/L at 25–30 °C (4.1–5.7 g NH_4^+-N/L, pH 7.5–7.8) [77,78], and 0.2–0.4 g NH_3-N/L at 35–38 °C (3.0–5.2 g NH_4^+-N/L, pH 7.6–7.9) [10,12,79,80]. At elevated temperature (51–64 °C, pH 7.4–7.9), digester inhibition has been concluded to commence at 0.6–1.5 g NH_3-N/L (2.5–11 g NH_4^+-N/L) [4,81] (calculations of ammonia or total ammonia were performed using Eq. (1) when required). However, digesters with ammonia-adapted microbial communities can still maintain operation at high ammonia concentrations. This adaptation has long been attributed to the methanogenic community, substantiated by observations of internal changes in dominant species and com-

munity shifts correlated to increased ammonia levels [4]. However, the numerous recent examinations of ammonia-stressed microbiomes highlighted in the previous section indicate that microbial ammonia adaptation should be complemented with development of a microbial community directing acetate conversion to methane through SAO. Our hypothesis is that a period of adaptation permits growth and establishment of the ammonia-tolerant SAOB and hydrogenotrophic methanogens able to remain active even under the high-ammonia conditions preceding the threshold for inhibition. Microbial community analyses of mesophilic processes, combined with determination of methane production pathway, indicate that the digester disturbance at ammonia levels exceeding 0.15 g NH_3-N/L can be a response to an ammonia-induced shift from acetoclastic methanogenesis to SAO and increased abundance of SAOB [11,12,68]. These studies, along with many others [13,16,18,25,35,82–84] have also shown dominance of the hydrogenotrophic pathway and/or high levels of hydrogenotrophic methanogens in correlation to high ammonia levels. Based on the results from several of these studies, the threshold for development of SAO as the dominant pathway for acetate conversion would probably be around 0.14–0.28 g NH_3-N/L at 37–38 °C (3.0–3.3 g NH_4^+-N/L, pH 7.5–8). To our knowledge, the actual threshold for a shift in pathway for acetate conversion in thermophilic methanogenic digesters has not yet been determined. However, in a defined thermophilic culture the shift from acetoclastic methanogenesis to SAO occurred at 0.03–0.05 g NH_3-N/L (0.7–1.4 g NH_4^+-N/L, pH 7.0, 55 °C) [48]. Screening analyses of industrial biogas processes have revealed similar ammonia levels (>0.14 g NH_3-N/L) for dominance of the SAO pathway at mesophilic temperatures, whereas in thermophilic conditions SAO is the main mechanism for acetate conversion at $\geqslant$0.24 g NH_3-N/L ($\geqslant$2.0 g NH_4^+-N/L) [2,13].

Within this context, the issue regarding eventual existence of an upper ammonia threshold for the functioning of an already ammonia-adapted process should be addressed. Several studies have reported a severe decrease in digester function and subsequent process failure of ammonia-adapted digesters at levels above 0.5–1.1 g NH_3-N/L (9.2–11.1 g NH_4^+-N/L, pH 7.1–7.8, 37–38 °C) [4,12,69,80]. Similarly to the first appearance of ammonia-induced digester disruption, this wide span most likely arises from differences in substrate composition, digester design and operating parameters, such as HRT and temperature [4]. As with the ammonia levels speculated to induce SAO, the diverse ammonia thresholds for severe inhibition could have a biological cause and could depend on the possibility for acclimatisation of the prevailing microbial community, or on the inoculum composition. However, the frequent dominance of SAO in digesters operating at and around the ammonia thresholds considered here clearly demonstrates the high ammonia tolerance of the syntrophic species and further indicates the importance of the activity of SAO populations and their interaction with the remaining anaerobic community for the functioning of the high-ammonia process.

It is conceivable that the actual ammonia level also affects SAO community structure. Ammonia-induced introduction of SAO and a concurrent shift in putative acetogenic community has namely been shown to be succeeded by a second alteration in community structure after a continuing rise in ammonia during anaerobic degradation [66,68]. Hypothetically, these dynamics could reflect microbial adaptations allowing SAO populations with higher ammonia tolerance to become dominant and thereby continue processing organic material despite the high ammonia levels.

4.2. Temperature and acetate concentration

Theoretically, SAO becomes energetically favourable at elevated temperature and acetate concentration [85]. Accordingly, SAO has appeared as the dominant pathway in a large number of thermophilic methanogenic systems (Table 1), supporting the hypothesis that higher temperature directly enhances the competitiveness of SAO relative to the acetoclastic pathway. In addition, temperature has a strong influence on the hydrogenotrophic methanogenic community structure [6,16,25,86–89], which in turn could affect the conditions for SAO. Another impact factor could of course be the temperature-induced increase in the NH_3 ratio, causing the level to exceed the ammonia threshold for increased contribution of the syntrophic pathway.

Dominance of SAO and presence of known SAOB in high-ammonia digesters operating within the mesophilic regime are frequently associated with elevated levels of acetate and propionate [10–13,27,57,106]. A possible source is direct ammonia inhibition of acetate- and propionate-degrading microorganisms, although to our knowledge the impact of ammonia specifically on syntrophic propionate communities has not been investigated to date. Another aspect is conceivably lower acetate conversion efficiency by SAO communities compared with the acetoclastic methanogens [45,106]. Accumulation of acetate could subsequently result in potential decreased propionate conversion rates [107], since the degradation of propionate follows the route of formation of acetate, carbon dioxide and hydrogen.

There is conflicting information about the influence of acetate concentration on the dominance of acetoclastic methanogenesis relative to the SAO pathway in complex microbial communities. Here, several different factors such as presence and structure of other acetate-degrading populations, the strains participating in SAO and the prevailing operating conditions, other than acetate, probably have a strong influence. Furthermore, SAO communities might employ a similar strategy to acetoclastic methanogens as regards acetate levels, i.e. with different types of microorganisms occupying unique niches based on diversified substrate affinity and growth rate. This theory is supported by genome-based analyses of *T. acetatoxydans* indicating a passive rather than an active acetate uptake system [108]. This species would consequently be favoured by high acetate concentrations, whereas the SAOB *S. schinkii* and *T. phaeum*, which most likely have active acetate uptake [109,110], could maintain activity at lower acetate concentrations. Increasing acetate concentrations has indeed been shown to stimulate growth (methane formation rate) in laboratory cultivation of defined syntrophic acetate-oxidising cultures at 30–46 °C. A requirement for a relatively high concentration of acetate (>25 mM) for methane formation has also been observed in defined microbial populations in a controlled environment [111].

In thermophilic continuous anaerobic digesters or in batch assays, SAO has been proposed as the predominant acetate degradation pathway at low acetate levels (0.2–1 mM) [94,112–114]. However favouring of SAO by high acetate concentrations (4–100 mM) has also been suggested [96,98,101]. In another batch study at thermophilic conditions, the syntrophic pathway was shown to dominate during incubation with initial acetate concentration of 250 mM and 6–7 g NH_4^+-N/L (0.09–2.67 g NH_3-N/L at 55 °C and pH 6.6–8.2), whereas the degradation was directed through acetoclastic methanogenesis at lower initial acetate levels (50 mM) [14]. The same research group also reported dominance of acetoclastic methanogenesis at acetate >1 mM and SAO at lower acetate levels [115]. The impact of acetate concentration on the conversion pathway in mesophilic temperature conditions has been less well examined. However, in long-term acetate-fed chemostats (ammonia level not specified) SAO dominated when 0.2 mM acetate was added, whereas acetoclastic conversion was detected when the digester was fed with 4 mM acetate [90]. Nevertheless, SAO has been proven to be the determinative metabolic pathway in semi-continuous mesophilic (37–44 °C) digesters with acetate concentrations ranging from >0.1 to 100 mM [10,12,16,57].

Table 1
Operating conditions and molecular investigations of anaerobic digesters (laboratory- or industrial-scale) and batch/enrichment cultures dominated by syntrophic acetate oxidation (SAO), verified by labelling experiments.[a]

Biological system[b]	Ammonia g NH_3-N/L (g NH_4^+-N/L)[c]	Operating parameters/experimental set-up[d]	Microbial community investigation[e]	
Mesophilic				
LS-CF	n/a	37 °C, pH 7 Acet: 0.01 g/L Dilution rate: 0.025/day	Quantitative RT-PCR of *mcrA* transcripts	[90]
IS-CF	4-5.6 g N/L	37–38 °C, pH n/a VFA: 1.8–2.7 g/L HRT: 20–25 days	FISH analyses of methanogens	[91]
LS-CF	0.6–1.0 (5.5–6.9)	37 °C, pH 7.9–8.0 VFA: 18–30 g/L HRT: 30 days OLR: 3 g VS/(L day)	qPCR analyses of methanogens and characterised SAOB, T-RFLP and clone library analyses of acetogenic communities (*fhs* gene), illumina amplicon sequencing of bacterial 16S rRNA genes	[11,68]
Batch	n/a	37 °C, pH 7.2–7.4 HRT: 30 days OLR 1.5 g COD/(L day)	MAR-FISH with ^{14}C-acetate, RNA-SIP with $^{13}C_6$-glucose and $^{13}C_3$-propionate to identify and quantify acetate-utilising communities	[71]
LS-CF	0.07–0.5 (1.5–11)	37 °C, pH 6.5–7.8 Acet: <0.1–16 g/L, prop: <0.1–10 HRT: 26–57 days OLR: 0.8–3.6 g VS/(L day)	qPCR analyses of methanogens and characterised SAOB	[12]
LS-CF with/without TE	0.3–0.5 (3.6)	37 °C, pH 7.9–8.1 Acet: 0.6–3.5 g/L, prop: 0.1–2.2 g/L HRT: 30 days OLR: 1.8–2.5 g VS/(L day)	qPCR analyses of methanogens and characterised SAOB	[57]
Batch	n/a	38 °C, pH 8.1 Acetate: 0.7 g/L		[92]
IS-CF	0.2–0.5 (3.3–4.9)	36–40 °C, pH 7.6–8.0 VFA: 3–13 g/L	qPCR analyses of methanogens and characterised SAOB	[13]
IS-CF	0.3–0.4 (2.9-4.6)	37–38 °C, pH 7.9 VFA: 0.6–0.8 g/L	FISH analyses of methanogens	[2]
LS-CF	0.2–0.3 (4.2–5.2)	35 °C, pH 7.5 VFA: 1 g/L OLR 2.2 g VS/(L day) 16–25% SAO contribution	Shotgun sequencing, DNA-SIP with ^{13}C-acetate and FISH-NanoSIMS analyses of bacterial communities	[15]
LS-CF with/without TE	0.4–1.5 (5.4–5.8)	37–42 °C, pH 7.9–8.1 Acet: <0.1–3.4 g/L, prop: <0.1–6.3 g/L HRT: 30 days sOLR: 2.3 g VS/(L day)	qPCR analyses of methanogens and characterised SAOB, T-RFLP and/or clone library analyses of bacterial (16S rRNA), acetogenic (*fhs* gene) and methanogenic (*mcrA* gene) communities	[16]
IS-CF	0.4–1.5 (2.4–4.2)	37–40 °C, pH 7.7–8.2 Acet: 0.3–0.5 g/L, prop: 0.005–0.02 g/L HRT: 21–32 days	Illumina amplicon sequencing of bacterial and archaeal 16S rRNA genes	[18]
Thermophilic				
Acetate enrichment	n/a	60 °C, pH 6.5–6.8 Acet: 3 g/L		[93]
Acetate chemostat		60 °C, pH 6.5–6.8 Acet: 0.6 g/L	Microscopic examinations	[94]
IS-CF	2.2–2.6 g N/L	52–55 °C, pH n/a VFA: 0.2–0.8 g/L HRT: 15–25 days	FISH analyses of methanogens	[91]
Batch	n/a	55 °C, pH ~ 7		[95]
Batch	n/a	55 °C, pH 6.8–7.8 Acet: 6 g/L BM		[96]
LS-CF	n/a	55 °C, pH 7.2 Acet: 0.1 g/L, prop: 0.07 g/L HRT: 4 days OLR 6.25 gCODcr/(L day)	Clone libraries and sequencing of bacterial and archaeal 16S rRNA genes	[50]
Batch	n/a	55 °C, initial pH 5.5 Acet: 6 g/L BM	qPCR analyses of methanogenic (16S rRNA) and acetogenic (*acsB* and *fhs* genes) communities	[97]
Batch	n/a	55 °C, pH > 7.5 Acet: 9–12 g/L BM	ARISA of archaeal and bacterial communities; qPCR analyses of acetogens (*acsB* and *fhs* genes)	[98]
LS-CF	0.7–1.0	55 °C, pH 6.7–7.0 Acet: 0.07–0.30 g/L COD, prop: 0.01–0.14 g/L COD HRT: 2–4 days	FISH analyses and 16S rRNA gene pyrosequencing of bacterial and methanogenic communities	[99]
Batch	0.06 (1)	53 °C, pH 7.3 Acet: 1 g/L BM	FISH analyses of archaeal community	[100]
Batch	0.09–2.7 (6–7)	55 °C, pH 6.6–8.2 Acet: 15 g/L	qPCR analyses of methanogens	[14]
IS-CF	0.2–0.8 (2.0–3.2)	48–55 °C, pH 7.7–8.1 VFA: 1.9–3.8 g/L HRT: 20–101 days OLR: 2.5–3.5 g VS/(L day)	qPCR analyses of methanogens and characterised SAOB	[13]

(*continued on next page*)

Table 1 (*continued*)

Biological system[b]	Ammonia g NH_3-N/L (g NH_4^+-N/L)[c]	Operating parameters/experimental set-up[d]	Microbial community investigation[e]	
IS-CF	0.5 (2.0–2.4)	52–55 °C, pH 7.9–8.0 VFA: 0.9–1.8 g/L	FISH analyses of methanogens	[2]
LS-CF	0.7–1.0 g NH_4^+/L	55–65 °C, pH 6.7–7.1 Acet: 0.06–2.1 g COD/L, prop: 0.04–0.6 g COD/L HRT: 2–4 days	454 pyrosequencing of bacterial and archaeal 16S rRNA genes	[74]
Batch	TAN 1.8 g/L	52 °C, pH 7.7 Acetate: 0.2–6 g/L	Proteome analyses	[101]
IS-CF	2.2–3.4 (0.7–1.5)	50–53 °C, pH 8.0–8.4 Acet: 0.05–1.6 g/L, prop: 0.01–0.2 g/L HRT: 3–15 days	Illumina amplicon sequencing of bacterial and archaeal 16S rRNA genes	[18]

n/a indicates not available.

[a] The following articles also proposed SAO as likely acetate degradation pathway but did not confirm dominance with labelling experiments [3,6,25,27–35,37,38,72,86,102–105].

[b] LS-CF laboratory-scale (semi) continuously fed digesters; IS-CF industrial-scale continuously fed digesters; TE addition of trace element mixture including iron.

[c] TAN total ammoniacal nitrogen.

[d] Acet – Acetate; VFA – volatile fatty acids; HRT – hydraulic retention time; OLR – organic loading rate; VS – volatile solid; COD – chemical oxygen demand; prop – propionate; BM – incubation in basal medium containing trace elements and vitamins.

[e] RT-PCR – reverse transcription PCR; *mcrA* – methyl coenzyme-M reductase; FISH – fluorescence in situ hybridization; qPCR – quantitative PCR; SAOB – syntrophic acetate oxidising bacteria; T-RFLP – terminal restriction fragment length polymorphism; *fhs* – gene encoding formyltetrahydrofolate synthetase; MAR-FISH – microautoradiography FISH; DNA-SIP – DNA stable carbon isotopic probing; NanoSIMS – nanometer scale secondary-ion mass spectrometry; *acsB* – gene encoding acetyl-CoA synthase; ARISA – automated ribosomal intergenic spacer analysis.

These results contradict acetate concentration as a determinative factor for SAO dominance in continuous anaerobic digesters. A mutual operating mode for the digesters studied was instead high ammonia level (>0.3 g NH_3-N/L), emphasising the strong influence of this parameter on the development of SAO. However, with regard to the influence of acetate concentration, it is important to bear in mind that even during nonappearance of acetate accumulation, the acetate formation rate can still be high within the anaerobic degradation process, as long as it does not exceed the consumption rate.

4.3. Influence of the methanogenic community structure

Syntrophic microorganisms strictly depend on the structure and activity of the methanogenic community and its efficiency in removal of hydrogen and/or formate [23]. Another potentially crucial impact factor is the competition for acetate exerted by the acetoclastic methanogens. Consequently, since parameters such as ammonia inhibition, temperature and acetate concentration strongly influence the methanogenic community structure, there is potentially an additional indirect effect on SAOB.

4.3.1. Acetoclastic methanogens

Methanosarcina generally exhibits higher growth rate but requires acetate concentrations above 1 mM, whereas *Methanosaeta* species typically dominate below that range, due to their higher affinity for acetate [116,117]. *Methanosaeta* sp. have low tolerance to specific inhibitors such as fluoroacetate and methyl fluoride, but also to free ammonia and high pH, possibly due to their restricted metabolic capability to use only the acetoclastic pathway [116]. Consequently, inhibitors for the acetoclastic methanogens most likely induce replacement of *Methanosaeta* with SAOB as the main acetate consumers in methanogenic systems. A further indication of this is that the level of *Methanosaeta* has been shown to be negatively correlated to high ammonia concentrations and dominance of SAO in biogas digesters [13,91].

The link between *Methanosarcina* and SAO is somewhat complicated. This methanogenic group can possibly act as a hydrogen scavenger [116] and their presence has been frequently reported at relatively high levels in investigations of several SAO-dominated processes and occasionally at relatively high ammonia levels [6,11,12,16,29,50,57,73,91,96]. In thermophilic batch cultivations *Methanosarcina* has actually been demonstrated to be able to catalyse acetoclastic methanogenesis even under high ammonia stress [115]. However, possible acetoclastic activity in long-term, continuously operating systems requires further research. In large-scale industrial digesters, the abundance of *Methanosarcinaceae* has been found to be negatively correlated with total ammonia concentration [22] and SAO dominance [13]. Another theory explaining the high abundance of *Methanosarcina* species in SAO-dominated systems is that they are able to mediate the entire process, i.e. both acetate oxidation and subsequent methanogenesis [57,91].

Interestingly, *Methanosaeta* and *Methanosarcina* have recently been demonstrated to accept electrons via direct interspecies electron transfer (DIET) in reduction of carbon dioxide to methane [118,119], which raises the possibility that SAO does not exclusively proceed via diffusion of electron carriers (hydrogen and formate) and that *Methanosaeta* could also be involved in SAO.

4.3.2. Hydrogenotrophic methanogens

A critical trait of a partner methanogen is the ability to maintain a sufficiently low hydrogen or formate concentration to make bacterial acetate oxidation thermodynamically feasible. The hydrogen partial pressure is thereby restrained within a low, narrow range [85], which has been and experimentally determined to range between 10–50 Pa [120,121] and 1.6–6.8 Pa [122] in thermophilic and mesophilic conditions, respectively. The acceptance of higher H_2 partial pressure for the implementation of SAO under thermophilic temperatures hypothetically includes *Methanosarcina* species as possible hydrogenotrophic partners at these higher temperatures. Certain species belonging to the mixotrophic *Methanosarcina* are able to reduce the H_2 partial pressure to >10 Pa. Methanogens more specifically specialised to use H_2 and CO_2 for growth cease oxidation of H_2 at slightly lower partial pressures, around 1–10 Pa [123], which is required for SAO at lower temperatures. Under pure culture conditions the methanogenic partners identified for SAO in thermophilic conditions have so far included *Methanobacterium* sp. and *M. thermautotrophicus* [39,40,121]. In thermophilic SAO digesters, *Methanomicrobiales* and *Methanobacteriales* (*Methanothermobacter*) are reported to be the dominant hydrogenotrophic methanogens [2,50,74,98]. *Methanomicrobiales*

and *Methanobacteriales* are likewise highly abundant in mesophilic SAO-dominated digesters [2,15]. In particular, *Methanoculleus* species [27,90] and *Methanoculleus bourgensis* have been emphasised as possible methanogenic partners in mesophilic SAO digesters [12,16,69,106]. *M. bourgensis* is reported to be the partner methanogen in SAO under pure co-cultivation in mesophilic conditions [42–44]. Adequate ammonia tolerance combined with high affinity for hydrogen may be the cause of *M. bourgensis* dominance in such conditions [124].

5. Deliberate operating strategies for SAO-mediated processes

The following section present examples of studies examining the impact of operating parameters, such as retention time, temperature, addition of trace elements and bioaugmentation, on syntrophic community structure and on the performance of SAO-mediated anaerobic digestion. The objective is to promote process optimisation through considering the activity and growth of this key microbial community.

5.1. Retention time, support material and organic loading

To avoid washout of the consortium, the operating solid retention time is recommended to exceed the microbial doubling time, which is worth considering in processes where methane formation is directed through SAO. The doubling time of acetoclastic methanogens, experimentally determined to be about 8–36 h and 1–9 days for *Methanosarcina* and *Methanosaeta*, respectively [125], can be compared with the 28 days obtained in cultivation of defined syntrophic acetate oxidising co-culture at 37 °C (initial acetate concentration 50 mM) [122]. Thermophilic SAO cultures have, however, displayed shorter doubling times of around 1.3–3 days (55–60 °C; initial acetate concentration 40–80 mM) [45], which indicates that the growth rate of certain SAO cultures could exceed the growth rate of *Methanosaeta*.

Increased HRT and immobilisation of microorganisms are proposed preventative actions against ammonia inhibition [3,4]. Accordingly, the relatively slow growth of the syntrophic co-cultures indicates that, in certain conditions, this would probably increase the prospects for establishment of the syntrophic microbes. However, there is a wide disparity in HRT in digesters in which SAOB have been detected. Species taxonomically related to *S. schinkii* have been observed in continuous biogas digesters operating at HRT ranging from 17 to 130 days, whereas *C. ultunense*, *T. acetatoxydans* and *P. lettingae* have been detected at HRT between 24–64, 24–101 and 40–60 days, respectively [6,11–13,16,17,51,55,57]. Bacteria related to *C. ultunense*, *T. phaeum* and *S. schinkii* have also been detected in the methane-producing digester of a two-stage system operating with a retention time of 8 days [29]. In thermophilic conditions (55 °C) and at low ammonia levels, successful operation of a SAO-dominated process may even be achievable at HRT down to 3 days [99]. Inclusion of support material or the formation of granular sludge, flocks or biofilms most likely support SAOB, since they can survive in the digester despite slow growth, but also since the distance between the bacteria and the hydrogen-consuming methanogen is reduced, which facilitates interspecies hydrogen transfer [108] or possibly DIET [126]. Accordingly, *T. phaeum* has been identified in a thermophilic upflow anaerobic filter reactor and biofilms with HRT of 1.6–21 days [49,53]. Overall, however, although HRT may be a decisive factor for development of SAO, other operating parameters such as digester configuration (e.g. recycling of digester sludge, presence of support material) and environmental conditions (e.g. ammonia concentrations and temperature) probably also have an influence.

The organic loading rate (OLR) is another important operating parameter in this context. OLR is known to impact on acetoclastic methanogenic structure [6,15,27,69,116], which possibly alters the conditions for SAO. OLR has also been revealed to influence the acetogenic community structure and population abundances, including that of potential SAOB, in biogas digesters operating under high ammonia and mesophilic conditions [69]. This suggests that operating parameters such as OLR influence the ability of different populations to grow and remain active within the anaerobic system. Consequently, promotion of highly efficient SAO populations could enable management to optimise biogas production (such as increased loading rate) even in high-ammonia digesters.

5.2. Addition of trace elements and iron oxides

In addition to high ammonia concentrations, the degradation of protein-rich substrates can also result in prevalence of high sulphide levels, which cause formation of metal-sulphide precipitates and thereby decrease the bioavailability of essential trace elements [7]. Addition of trace elements may therefore be considered exceptionally beneficial in the anaerobic degradation of protein-rich materials. Inclusion of iron in the trace element additive may also enhance the positive effects, since sulphide precipitation of trace metals is constrained, due to the primary removal of sulphide by the iron [3,7]. Furthermore, presence of conductive iron oxides (e.g. magnetite and hematite) has been shown to accelerate syntrophic oxidation of acetate and propionate to methane in methanogenic sludge [73,127,128]. This suggests that supplementation with iron particles may be an interesting approach for possible acceleration of VFA degradation and performance improvements in high-ammonia digesters.

Addition of trace elements to high-ammonia mesophilic digesters (>3.0 g NH_4^+-N/L; 0.14 g NH_3-N/L at 35–37 °C), dominated [16,57], or indicatively dominated [19] by the SAO pathway has been shown to substantially increase methane yield and restrict VFA accumulation. The level and composition of the trace element additive required for process optimisation depend on substrate and operating conditions. However, in high-ammonia, and therefore most likely SAO-dominated, industrial commercial biogas production systems, sufficient bioavailability of cobalt (0.5 mg/L) and nickel (0.2 mg/L) has been proven to be highly important for good performance of the processes [129]. Ortner et al. [130] suggested considerably higher levels of bioavailable cobalt (15.7 mg/L) and nickel (7.1 mg/L), combined with molybdenum (3.2 mg/L) for optimum process performance. However, using such high levels would obstruct the use of the digestion residue as a fertiliser on arable land.

Surprisingly, quantitative analyses have demonstrated comparable [57] or lower abundance [16] of known SAOB in digesters with a trace element additive, despite persistent dominance of SAO. In Karlsson et al. [57], *Methanosarcinales* was found to be the methanogenic group most favoured by trace element addition. Banks et al. [19] observed high abundance of *Methanomicrobiales* in high-ammonia digesters whether trace element was added or not. In Westerholm et al. [16], a methanogenic community characterised by a high proportion of *M. bourgensis*, with high population richness, was suggested as a resilient promoter for the enhanced performance of the digester receiving a trace element additive. The high hydrogen affinity of *M. bourgensis* have been mentioned previously in this review and comparable lower hydrogen partial pressure was also revealed in the high-performing trace element supplemented digester [16]. Interestingly, the response within the *M. bourgensis* structure has been shown to differ depending on the composition of the trace element additive [131]. Since low

hydrogen level is of particular importance in SAO-mediated anaerobic degradation, a change in hydrogen-utilising community structure, and thereby the prevailed hydrogen partial pressure, could possibly have a major impact in such systems.

5.3. Temperature

Elevated temperature extends the window of opportunity of SAO, meaning that the level of hydrogen or formate does not need to be reduced as much as is required at lower temperature. Consequently, methanogenic communities that remove hydrogen/formate less efficiently can act as SAO methanogenic partner. Hypothetically, such communities could exhibit higher growth rates and consequently allow operation at shorter HRT without digester disturbance compared with the methanogenic communities prevailing in mesophilic systems. Nevertheless, since higher temperature increases the proportion of NH_3, thermophilic digesters commonly allow operation at lower loads of nitrogen-containing materials, compared with mesophilic digesters. For that reason, several Swedish commercial biogas plants have reduced the operating temperature from 55 to 52 °C in order to enable functional operation (Malmros P., Uppsala Vatten AB and Moestedt, J., Tekniska Verken AB, personal communications).

Within the thermophilic spectrum, increasing the temperature from 55 °C to 65 °C has been shown to increase the contribution of methane generation via SAO from 60% to 100% [74]. Digester operation at somewhat enhanced mesophilic temperature of around 42–44 °C, with the aim of accelerating acetate conversion without excessively enhancing the ammonia ratio, suggests potential for improved functionality of SAO-dominated processes. This has proven to be a management strategy with a positive impact on high-ammonia digester performance (12% increased methane yield) during degradation of sulphur- and nitrogen-rich thin stillage [6]. In contrast, processing household waste in co-digestion with albumin at high ammonia at 42 °C instead of 37 °C had no, or even a negative, impact on performance of SAO digesters, operating with or without trace element addition [16].

5.4. Bioaugmentation

Continuous bioaugmentation of a natural mesophilic biogas-producing consortium by a defined SAO culture, comprising *C. ultunense*, *S. schinkii* and *T. acetatoxydans* and the hydrogenotrophic *M. bourgensis* sp. MAB1 has been assessed as a possible method to accelerate the adaptation period to gradually increasing ammonia levels [12]. Bioaugmentation had no significant effect on the abundance of *S. schinkii*, whereas elevated levels of *C. ultunense* and *T. acetatoxydans* were observed. However, the endogenous SAOB community rapidly increased in abundance in association with a shift from acetoclastic to acetate-oxidation (at >0.2 g NH_3-N/L), in the control digesters without bioaugmentation. Consequently, under the conditions studied, the abundance of SAOB did not appear intermediate for development of SAO as the dominant pathway for methane formation. Instead, this result indicates strong dependence on certain operating condition(s), most likely high ammonia concentration, high acetate concentration and/or the methanogenic community structure, for the dynamic transition to SAO, with concurrent increased abundance of the microorganisms involved [12]. In another study, bioaugmentation with *C. ultunense* and *M. bourgensis* sp. MAB1 was reported to have no observable impact on the microbial community or the function of a mesophilic, high-ammonia upflow anaerobic sludge blanket (UASB) reactor [132]. Instead, addition of a pure methanogenic culture (*M. bourgensis*, strain $MS2^T$) has been proposed to successfully enhance methane yield and increase the abundance of this species in an ammonia-stressed continuous biogas digester [133]. This provides additional evidence of the importance of *M. bourgensis* (discussed in the previous section) for optimised performance of SAO-dominated mesophilic processes.

6. Conclusions

The current intense discussion about global warming emphasises the need for efficient production of renewable energy. The formation of biogas from protein-rich biological material is advantageous in several regards, but the issues related to ammonia inhibition demand deliberated process operating strategies. High ammonia level directs the methanisation of acetate through the SAO pathway and ammonia-induced adaption of the biogas-producing consortium has been shown to involve dynamically changing microbial communities (including SAOB, acetogens and methanogens). A well-adapted process can cope with substantially higher ammonia levels than an unadapted process, an effect most likely associated with the capacity for development of an ammonia-tolerant microbial community that efficiently degrades acetate through the syntrophic pathway. Besides high ammonia level, acetate concentration, temperature and methanogenic community structure are also factors believed to shape and influence SAO-mediated microbial ecosystems.

Commercial biogas production may sometimes be on the border of economic feasibility and improved biogas yield, stabilisation of digester operation and increased value of the residual product would considerably increase interest in construction of commercial biogas plants. HRT, addition of trace elements, temperature and bioaugmentation are operating parameters that have been researched as strategies to improve the SAO process. Acetate conversion, mainly directed via SAO, has been demonstrated in digesters operating under a wide range of HRT. To our knowledge, no published study has so far considered HRT as a sole factor, which obstructs analyses of its influence on the syntrophic pathway. However, microorganisms involved in SAO are clearly able to remain active and competitive at a wide range of HRT. Recent high-ammonia studies regarding the impact by OLR and addition of trace elements indicate the potential to direct, create and manage microbial communities to optimise process performance. In line with this, both OLR and addition of trace elements have been shown to influence methanogenic and acetogenic community structures, including potential SAOB. Operation at higher temperature increases the probability of SAO development, possibly due to thermodynamic favouring of SAO by the higher temperature, and increased NH_3 ratio and successive inhibition of the acetoclastic methanogens competing for acetate. Bioaugmentation with syntrophic co-cultures does not facilitate the dynamic transition from acetoclastic methanogenesis to SAO, whereas addition of *M. bourgensis* improves adaptation to gradually increased ammonia in mesophilic conditions.

Considering the functional importance of syntrophic bacteria in methanogenic systems, increased knowledge of these populations is essential for forecasting process failures and for devising strategies for process optimisation. Next-generation sequencing technologies enable characterisation of complex microbial communities and, considering the rapid development within this area, the potential for targeting low-abundance populations, such as syntrophic communities, will increase. This area thereby holds great potential to expand knowledge of the influence of factors such as availability of nutrients, temperature and ammonia level, and consequently allow for more perceptive predictions of their behaviour in ecosystems. However, just as in interpretation and description in all microbial ecology, the challenge is of course to correlate the genetic data with functional traits. Analyses of the recently published genomes of *C. ultunense*, *T. phaeum*, *S. schinkii* and *T. acetatoxydans* could provide insights into functional genes

that can be related to the metabolic commitment, and enable inference of syntrophic entities present and their function within the community.

Acknowledgements

The authors gratefully acknowledge financial support from Microdrive, Formas, Norwegian Research Council and Tekniska verken i Linköping AB.

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