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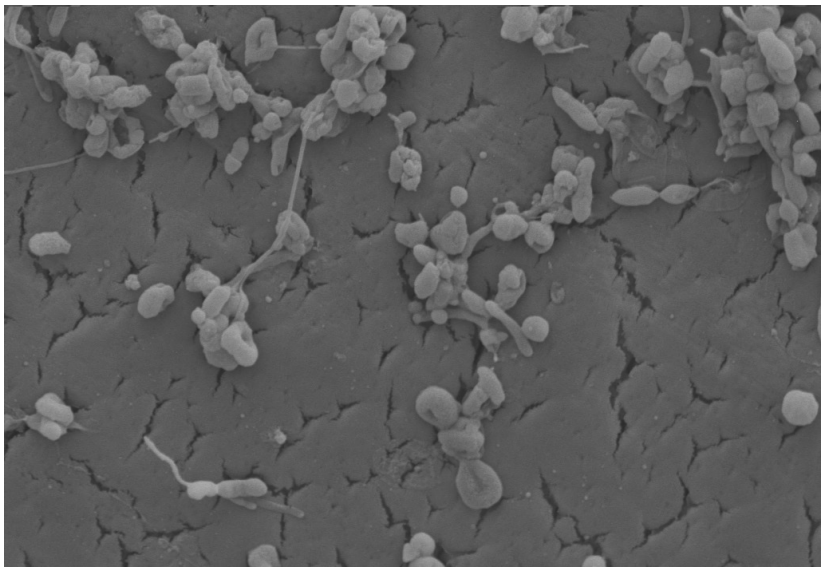
Optimisation strategies for ammoniatolerant syntrophic propionate-oxidising microorganisms

Effects of additives, ammonium levels and temperature on biogas production

Eduardo Pinela



Optimisation strategies for ammonia-tolerant syntrophic propionate-oxidising microorganisms

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Eduardo Pinela

Faculty of Natural Resources and Agricultural Sciences

Department of Molecular Sciences

Uppsala

SLU

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Cover: Scanning electron microscopy (SEM) photograph of microbial aggregates of a syntrophic propionate-oxidising enrichment culture on the surface of a phosphate precipitate (photo taken by Anna Neubeck, 2022)

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Abstract

Biogas production is a promising technology for cleaner energy production. It converts biodegradable waste in anaerobic reactors into two products: biogas and digestate. However, industrial biogas processes often face challenges, particularly ammonia inhibition during the anaerobic degradation of protein-rich materials. This inhibition causes ammonia-tolerant syntrophic microorganisms to become the main consumers of intermediates such as propionate and acetate, breaking them down into H_2 and CO_2, which hydrogenotrophic methanogens use to produce biogas. These microorganisms rely on each other for propionate and acetate degradation, a biological interaction known as syntrophy.

Yet, syntrophic microorganisms are often constrained in biogas processes, requiring optimisation strategies to enhance their activity. This is crucial for resilient biogas production processes operating under high-ammonia conditions.

This thesis explored optimisation strategies for syntrophic propionate and acetate degradation and hydrogenotrophic methanogenesis. It focused on adding various materials or iron and sulfur species, and examined the effects of varying levels of ammonia, zeolites, and operating temperatures. The results demonstrated the improvement of propionate and acetate degradation, and increasing methane production rates with the addition of several materials and iron and sulfur species, while also showing the cultures' sensitivity to changes in temperature, ammonia concentrations and high levels of certain iron and sulfur species. These findings suggest that such approaches can positively impact industrial biogas processes, but achieving maximum yield requires careful consideration of ammonia content, temperature, and microbial community structure during their implementation.

Keywords: biogas, syntrophy, propionate and acetate oxidation, hydrogenotrophic methanogenesis, temperature, ammonia inhibition, zeolite, iron and sulfur.

Optimeringsstrategier för ammoniak-toleranta syntrofa propionat-oxiderande mikroorganismer

Effekter av tillsatser, ammoniumnivåer och temperatur på biogasproduktion

Sammanfattning

Biogasproduktion är en lovande teknologi för renare energiproduktion. Den omvandlar biologiskt nedbrytbara avfall i anaeroba reaktorer till två produkter: biogas och rötrest. Dock möter industriella biogasprocesser ofta utmaningar, särskilt ammoniakhämmning under den anaeroba nedbrytningen av proteinrika material. Denna hämning gör att ammoniaktoleranta syntrofiska mikroorganismer blir de huvudsakliga konsumenterna av intermediärer som propionat och acetat, och bryter ner dem till H_2 och CO_2, som väteotrofiska metanogener använder för att producera biogas. Dessa mikroorganismer är beroende av varandra för nedbrytning av propionat och acetat, en biologisk interaktion känd som syntrofi. Ändå är syntrofiska mikroorganismer ofta begränsade i biogasprocesser, vilket kräver optimeringsstrategier för att förbättra deras aktivitet. Detta är avgörande för resilienta biogasproduktionsprocesser som verkar under höga ammoniakförhållanden.

Denna avhandling undersökte optimeringsstrategier för syntrofisk propionat- och acetatnedbrytning samt hydrogenotrof metanbildning. I studierna tillsattes olika material eller järn- och svavelföreningar och effekterna av varierande ammoniaknivåer, zeoliter och driftstemperaturer undersöktes. Resultaten visade på förbättrad nedbrytning av propionat och acetat samt ökade metanproduktionshastigheter med tillsats av flera material och järn- och svavelföreningar, samtidigt som det också visades att kulturerna var känsliga för förändringar i temperatur och ammoniaknivåer samt för höga halter av järn eller svavelföreningar. Därmed tyder dessa studier på att dessa strategier kan ha en positiv inverkan på industriella biogasprocesser, med hänsyn till ammoniakhalt, temperatur och mikrobiell samhällsstruktur för maximal avkastning.

Nyckelord: biogas, syntrofi, propionat- och acetatoxidation, hydrogenotrof metanogenes, temperatur, ammoniakinhibering, zeolit, järn och svavel.

Estratégias de optimização para microrganismos oxidadores de propionato tolerantes ao amoníaco

Efeitos de aditivos, níveis de amónio e temperatura na produção de biogás

Resumo

A produção de biogás é uma tecnologia promissora para a produção de energia mais limpa, convertendo resíduos biodegradáveis depositados em reatores anaeróbios em dois produtos: biogás e digerido. No entanto, os processos de produção industrial de biogás enfrentam frequentemente desafios, em particular a inibição por amoníaco durante a degradação anaeróbia de materiais ricos em proteínas. Esta inibição faz com que microorganismos sintróficos tolerantes ao amoníaco se tornem os principais consumidores de produtos intermédios como propionato e acetato, degradando-os em H_2 e CO_2, que os metanogéneos hidrogenotróficos utilizam para produzir biogás. Estes microorganismos dependem uns dos outros para a degradação do propionato e do acetato, uma interação biológica conhecida como sintrofia. No entanto, os seus metabolismos são frequentemente limitados nos processos de produção de biogás, exigindo estratégias de optimização para melhorar a sua atividade, algo essencial para processos de produção de biogás resilientes a condições de elevado teor de amoníaco.

Esta tese explorou estratégias de optimização da degradação sintrófica de propionato e acetato, bem como da metanogénese hidrogenotrófica. Focou-se na adição de vários materiais ou compostos de ferro e enxofre, bem como nos efeitos de diferentes níveis de amoníaco, zeólitos e temperaturas. Os resultados demonstraram a otimização das taxas de degradação do propionato e do acetato bem como da produção de metano através da adição de vários materiais e compostos de ferro e enxofre, ao mesmo tempo que evidenciaram a sensibilidade das culturas a variações de temperatura e níveis de amoníaco. Assim, estes estudos sugerem que estas estratégias podem ter um impacto positivo nos processos industriais de produção de biogás, sendo necessário ter em consideração o teor de amoníaco, a temperatura e a estrutura da comunidade microbiana para obter ganhos máximos.

Palavras-chave: biogás, sintrofia, oxidação de propionato e acetato, metanogénese hidrogenotrófica, temperatura, inibição por amoníaco, zeólitos, ferro e enxofre.

Preface

This thesis aims to propose and demonstrate feasible optimisation strategies for the syntrophic degradation of propionate and acetate, alongside methane production, in industrial biogas processes where ammonia-tolerant syntrophic microorganisms flourish. It is a valuable resource for biogas plant operators and environmental microbiologists, providing insights into the microbiological relationships among these microorganisms, how they are impacted by environmental parameters and how their metabolisms can be enhanced to overcome the thermodynamic barriers of the reactions they catalyse.

Dedication

To my family and loved ones, you are and will always be my reason for being here.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Eduardo Pinela, Anna Schnürer, Anna Neubeck, Jan Moestedt, Maria Westerholm (2024). Impact of additives on syntrophic propionate and acetate enrichments under high-ammonia conditions. Applied Microbiology and Biotechnology, 108.1 (433), https://doi.org/10.1007/s00253-024-13263-7

II. Eduardo Pinela, Jan Moestedt, Maria Westerholm (2025). Synergistic effects of zeolite, ammonium and temperature on propionate degradation and methane generation by ammonia-tolerant syntrophic microorganisms. (submitted)

III. Eduardo Pinela, Maria Westerholm (2025). Optimisation of propionate oxidation and methane production in thermophilic ammonia-tolerant syntrophic cultures: effects of ammonium levels, temperature, and zeolite dosage. (manuscript)

IV. Eduardo Pinela, Sepehr Shakeri Yekta, Jan Moestedt, Maria Westerholm (2025). Improving syntrophic propionate degradation in ammonia-tolerant enrichment cultures and sludges through the addition of iron and sulphur compounds. (manuscript)

Paper I is reproduced with the permission of the publishers.

The contribution of Eduardo Pinela to the papers included in this thesis was as follows:

I. Contributed to designing the experiments, conducted the experiments and most lab work, performed data analysis and statistical analysis, and wrote the original manuscript draft.

II. Contributed to designing the experiments, conducted the experiments and most lab work, performed data analysis and statistical analysis, and wrote the original manuscript draft.

III. Contributed to designing the experiments, conducted the experiments and most lab work, performed data analysis and statistical analysis, and wrote the original manuscript draft.

IV. Contributed to designing some experiments (addition of iron and sulfur compounds to anaerobic digestion of sludges), conducted some experiments (addition of iron and sulfur compounds to syntrophic mesophilic enrichments and anaerobic digestion of sludges), performed the respective lab work, collected the data and performed the statistical analyses, and wrote the original manuscript draft.

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Abbreviations

AD	Anaerobic digestion
AM	Acetoclastic methanogens
CM	Conductive material
DIET	Direct interspecies electron transfer
GHG	Greenhouse gas
HM	Hydrogenotrophic methanogens
HRT	Hydraulic retention time
IET	Interspecies electron transfer
MMC	Methylmalonyl-CoA
OLR	Organic loading rate
SAO	Syntrophic acetate-oxidation
SAOB	Syntrophic acetate-oxidising bacteria
SEM	Scanning electron microscopy
SPO	Syntrophic propionate-oxidation
SPOB	Syntrophic propionate-oxidising bacteria
SRB	Sulfate-reducing bacteria
VFA	Volatile fatty acids

1. Introduction

The Industrial Revolution paved the way for significant technological advancements and the exploration of environments and resources to an extent never before realised. The mechanisation and systematisation of exploration and production processes facilitated sustained population growth and improved living standards in many Western countries (Frader 2006). However, these combined factors also led to increased production demands for both basic and non-essential products, often resulting in greater environmental impacts. Anthropogenic activities such as burning fossil fuels for energy production, deforestation for agriculture and livestock farming, the industrial manufacturing of fossil-fuel-based chemicals like plastics and fertilisers, and inadequate waste management strategies have significantly contributed to the exponential rise in greenhouse gas (GHG) emissions observed over the past 200 years. As a result, higher planetary temperatures and more frequent extreme weather events are becoming apparent (IPCC 2021). Thus, climate change is a reality we face today more than ever, and actions are required to mitigate the effects of polluting anthropogenic activities. To achieve this, effective and sustainable strategies for energy and food production must be developed to replace current unsustainable practices.

One such strategy is implementing anaerobic digestion (AD) of organic waste streams to produce biogas and digestate (Figure 1). AD for biogas production is a well-known technology that has been explored for centuries (Marchaim 1992). Its products, biogas and digestate, can serve as substitutes for fossil fuels (Ryckebosch et al. 2011) and fossil-derived fertilisers (Hagman and Eklund 2016), respectively. Furthermore, biogas production assists societies in mitigating GHG emissions by promoting the selective

recovery of organic wastes that would otherwise end up in lanfills (Cherubini et al. 2009).

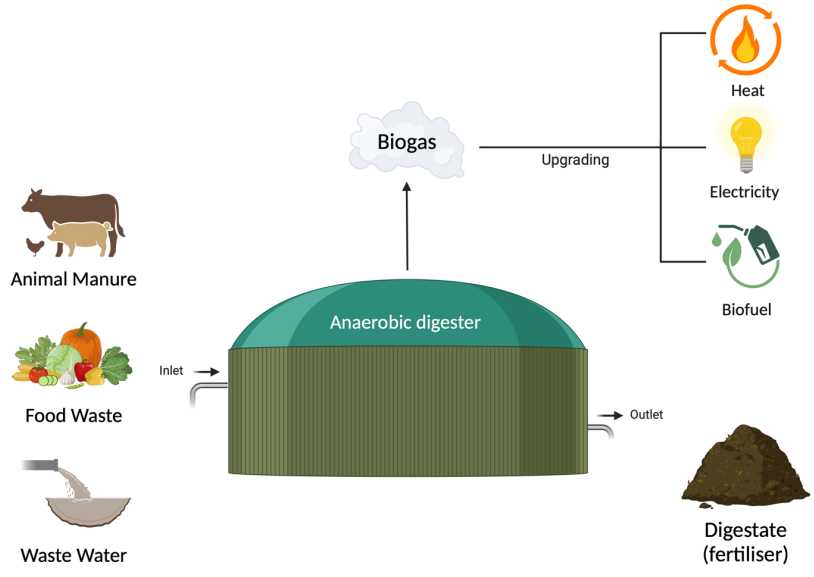

Figure 1. The biogas production schematic pathway, its end products and applications.

However, due to the diversity of organic waste streams and the complexity of the process, numerous challenges may arise during the operation of such an AD system. One of the most prevalent challenges is the accumulation of ammonia (NH_3), a process inhibitor that originates from the breakdown of protein-rich materials. This accumulation hinders several steps of AD (Yenigün and Demirel 2013), which rely on the coordinated activity of various microbial groups that must cooperate in a balanced manner for an effective process to occur (Schnürer and Jarvis 2018). When NH_3 levels become excessively high, this balance is disrupted, leading to the accumulation of intermediate compounds like the volatile fatty acids (VFA) propionate and acetate. This is highly undesirable since it can lead to losses in methane potential, a greater risk of methane emission during the post-storage of digestate, overall reduced process efficiency, and potentially complete process failure (Chen et al. 2008, Hao et al. 2017, Li et al. 2017, Shi et al. 2017, Zhang et al. 2018). Under these conditions, the activity of ammonia-tolerant, syntrophic microbial species such as syntrophic

propionate-oxidising bacteria (SPOB), syntrophic acetate-oxidising bacteria (SAOB), and hydrogenotrophic methanogens (HM) becomes crucial for successful AD performance (Fotidis et al. 2014, Hao et al. 2017, Singh et al. 2023). However, strategies to specifically improve syntrophic propionate and acetate degradation and methane production have not yet been addressed, as syntrophic microorganisms can be difficult to study in complex anaerobic communities. This is because syntrophic microorganisms are frequently found in low abundance (Guo 2024), grow slowly, possess low metabolic rates (Koster and Koomen 1988, Ito et al. 2012, Westerholm et al. 2019, Jannat et al. 2021), and their interconnected metabolisms can be difficult to optimise (Schink 1997). Nonetheless, their significance for the stability of biogas production processes, particularly those functioning at high-ammonia conditions, is crucial for the system's performance and resilience. Therefore, enhancing the understanding of their cooperation and uncovering novel methods to specifically improve these microorganisms' activity is of great importance.

This thesis aims to provide new insights into optimisation strategies for syntrophic propionate and acetate oxidation and methane production in biogas production processes under high ammonia levels. To address the challenges of studying syntrophic activities within complex communities, ammonia-tolerant syntrophic enrichment cultures were used. Specifically, the presence of supportive materials and various environmental conditions were examined to enhance syntrophic activity, including the reduction of lag phases and the increase in propionate and acetate degradation, along with methane production rates. The specific objectives of the different articles (I-IV) are as follows:

- To evaluate the impact of additives on syntrophic activity and the aggregation of syntrophic species under mesophilic and thermophilic conditions (I, II, III);
- To determine synergistic effects of temperature, ammonium (NH_4^+) levels and zeolite dosages on syntrophic activity under mesophilic and thermophilic conditions (II, III);
- To assess the effect of iron and sulfur compounds on syntrophic activity under mesophilic and thermophilic conditions (IV).

2. Anaerobic digestion for biogas production

2.1 Anaerobic digestion for energy and nutrient production

AD of organic waste is a biotechnological process that generates high-value compounds, namely biogas, and a sustainable fertiliser (digestate) (Figure 1). Biogas is a renewable energy source that can be used to replace fossil fuels. Replacing gasoline with biogas as fuel for a mid-size car, for instance, can reduce GHG emissions from 49.6 to 9.4 tons of total CO_2-equivalent life-cycle emissions (Buberger et al. 2022), thus aiding in mitigating their environmental and societal impacts. Biogas typically consists of 50-75% CH_4 and 25-50% CO_2 and can be directly used in combined heat and power units, upgraded to biomethane (typically 95-97% CH_4) for gas grid injection, employed in industrial applications, or used as gaseous or liquefied vehicle fuel (Ryckebosch et al. 2011, Hamzehkolaei and Amjady 2018). Digestate is a valuable product from the AD of organic wastes as it can be used as a sustainable fertiliser, promoting the recirculation of nutrients from organic matter back into the soil (Hagman and Eklund 2016, Tampio et al. 2024) (Figure 1). When used as a fertiliser, digestate can substitute fossil-derived fertilisers, further contributing to the mitigation of GHG emissions while achieving similar agricultural yields (Jin et al. 2022). For instance, Kowalczyk-Juśko et al. (2023) showed that the use of different digestates as replacements for mineral fertiliser can reduce GHG emissions by 27.9-61.6 kg CO_2-equivalent per ton of digestate. Additionally, the biogas production process is also a highly efficient and environmentally friendly waste management technology, as it promotes a selective recovery and treatment of organic wastes in a controlled environment where the resulting GHG are captured and stored (Rama et al. 2023). These organic wastes would

otherwise be disposed of in landfills or incinerated, increasing GHG emissions (Cherubini et al. 2009).

AD can be applied to various substrates, each with different chemical compositions and methane potentials, resulting in different degradation efficiencies, biogas yields, nutritional value of digestates and presenting unique technological and process challenges (Carchesio et al. 2014, Müller-Stöver et al. 2016, Niemiec et al. 2022, Tampio et al. 2024). Carbohydrate-rich materials are among the most abundant substrates; however, fully harnessing their biogas potential is challenging due to their low degradability, particularly in the case of lignocellulosic substrates (such as forest waste and plant materials). To optimise the biogas potential of these materials, various pre-treatment techniques, including steam explosion and enzymatic treatments (Ziemiński et al. 2012, Steinbach et al. 2019, Hashemi et al. 2021), can be employed, although the economic feasibility of these methods on an industrial scale remains limited (Ahmad et al. 2018). Substrates demonstrating higher methane potential include lipid-rich materials such as vegetable oils and other cooking oils, as well as protein-rich substrates like animal manure, slaughterhouse waste, and food waste (Labatut et al. 2011). However, supplying the biogas process with these materials can present challenges related to their breakdown products. A common issue arising from the treatment of lipid-rich substrates is long-chain fatty acid inhibition (Rasit et al. 2015). Long-chain fatty acids, produced during lipid breakdown in the substrate, inhibit the process by reducing microbial access to the substrate through adsorption onto the cell walls of the bacteria involved in the AD process (Hwu et al. 1998). Regarding AD processes treating protein-rich substrates, a problem that frequently arises is NH_3 inhibition (Sun et al. 2016). NH_3 is generated from protein breakdown, and while the mechanism of its inhibitory action remains poorly understood, it has been hypothesised that this molecule may passively diffuse through cell membranes, creating an internal proton imbalance that microorganisms must compensate for at an energy cost (Kayhanian 1999). Nevertheless, protein-rich substrates such as animal manure, slaughterhouse waste, and food waste not only demonstrate high methane potential but also yield high-quality digestates suitable for use as biofertilisers (Al Seadi and Lukehurst 2012). Despite the challenges associated with the AD of these and other substrates, several operational strategies can be implemented to mitigate these issues.

2.1.1 Common operating parameters

AD systems may have different configurations that vary in both the technology of the reactors and the operating parameters, such as temperature and pH (Rocha-Meneses et al. 2022). Such systems can be set as a batch, where the substrate is introduced once into the system and the degradation proceeds until the desired methane yield is achieved. The digestate is then removed, and the process is reset for a new cycle. However, more commonly, these systems function as continuous systems, where the substrate is fed into the system continuously at a specific rate (organic loading rate (OLR)), allowed to degrade for a designated period (hydraulic retention time (HRT)), and then removed from the system (Chowdhury and Fulford 1992). Parameters like substrate composition, OLR and HRT must be balanced to prevent process disturbances such as increased concentrations of toxic compounds or microbial washout (Pera et al. 2022). These systems can be designed as single-stage processes, where all steps of substrate degradation occur within the same digestion tank, or as two- or multi-stage processes, where the initial steps of AD occur in one tank, and subsequent steps take place in another tank (Perman et al. 2022, Yang et al. 2024).

The process temperature and the type of substrate being degraded are particularly important considerations when selecting the system setup. AD can operate within a range of temperatures, with 37-42°C (mesophilic) (Marañón et al. 2001, Nasir et al. 2012) and 50-55°C (thermophilic) (Abouelenien et al. 2016, Maus et al. 2016, Perman et al. 2024) being the most commonly employed in industrial biogas production processes. Thermophilic conditions can enhance overall degradation and biogas production rates by increasing the solubility of organic compounds and accelerating the metabolism of microorganisms (Singh et al. 2023). Nevertheless, operating at higher temperatures also poses greater risks for process instability, as there is a heightened potential for process inhibition by toxic compounds and a reduced ability to adapt to process changes due to decreased microbial diversity (Hao and Wang 2015, Ao et al. 2021). Consequently, many biogas plants operate their processes under mesophilic conditions, which, despite exhibiting lower degradation rates and biogas production, are more stable due to their higher microbial diversity and lower risk of rapid inhibition from toxic compounds (Niu et al. 2015, Gebreeyessus and Jenicek 2016).

2.1.2 Ammonia inhibition of the biogas process and common operating strategies to mitigate disturbances

Ammonia (NH_3) and its ion, ammonium (NH_4^+), exist in equilibrium in solution. This compound is an important nitrogen source for microbial growth; however, when present in high levels, NH_3 inhibits several microbial species involved in the degradation steps of the AD process (Yenigün and Demirel 2013). Both temperature and pH play a role in shifting the reaction, with higher temperatures and pH levels contributing to a greater occurrence of NH_3, the most toxic of the two compounds (Kayhanian 1999). Other process parameters, such as OLR and HRT, also impact NH_3 inhibition, with high OLRs and extended HRTs further increasing NH_3 inhibition of processes degrading protein-rich materials. This occurs because higher OLRs increase the concentration of proteins available to the microorganisms involved in AD, resulting in elevated NH_4^+ levels when the proteins are degraded (Magdalena et al. 2020, Song et al. 2022). However, too high OLRs can result in overload, leading to decreased microbial activity, consequent reduced protein degradation and, hence, lower NH_4^+ production (Nkemka et al. 2015). In turn, longer HRTs allow microorganisms more time to further degrade the proteinaceous materials, maintaining the generated NH_4^+ in the system for a longer period and increasing the level of NH_3 inhibition (Alepu et al. 2016, Magdalena et al. 2020, Kalamaras et al. 2021). Thus, one common approach to address NH_3 toxicity is to decrease the protein content of the ingoing substrate, as this lowers the protein concentration, which in turn reduces the concentration of the formed NH_3 and NH_4^+. This can be achieved by incorporating more carbon-rich substrates into the substrate mix (Lansing et al. 2019), thereby increasing the carbon-to-nitrogen (C:N) ratio. This strategy dilutes the concentration of protein material in the incoming substrate, subsequently reducing the production of NH_3 and NH_4^+ while still providing sufficient carbon as energy sources for the microbial communities (Zeshan et al. 2012). Another common strategy that allows for reducing the level of NH_3 toxicity is by performing ammonia stripping: either side-stream stripping, which is done to a portion of the digester's liquid during digestion, or a stripping of the digestate that is going to be recirculated (Limoli et al. 2016, Li et al. 2018). The most common method, air stripping, relies on the chemical reaction balance between NH_4^+ and NH_3 (gas) and on the fact that, by increasing the temperature and the pH, the reaction is pushed towards the production of the latter. As NH_3 is a gas, it can be physically separated and

chemically recovered from the liquid waste stream, with the treated liquid waste stream then being returned to the digester (Di Capua et al. 2021). However, removing NH_3 from the system will also result in a lower NH_4^+ content in the digestate, which is an important component of the added value of digestates as fertilisers, as this is the plant-available nitrogen source (Sørensen et al. 2011). To maintain the NH_4^+ content in the digestate, an interesting optimisation strategy that is operational but relies on its impacts on the microbial community structure is the acclimatisation of the microbial community to high levels of NH_3. By enabling the microbiota to adapt to elevated NH_3 levels, it is feasible to operate biogas production processes under these conditions, as several processes do (Fuchs et al. 2018). Nonetheless, adjustments must be made to the operational parameters to accommodate the specific biological needs of the resulting microbial communities.

2.2 Microbiology of the AD process

In the AD process, the substrate is subjected to the activity of various groups of microorganisms, which sequentially degrade complex organic compounds into CH_4 and CO_2 through four main stages (Chen and Neibling 2014, Lohani and Havukainen 2018, Schnürer and Jarvis 2018) (Figure 2).

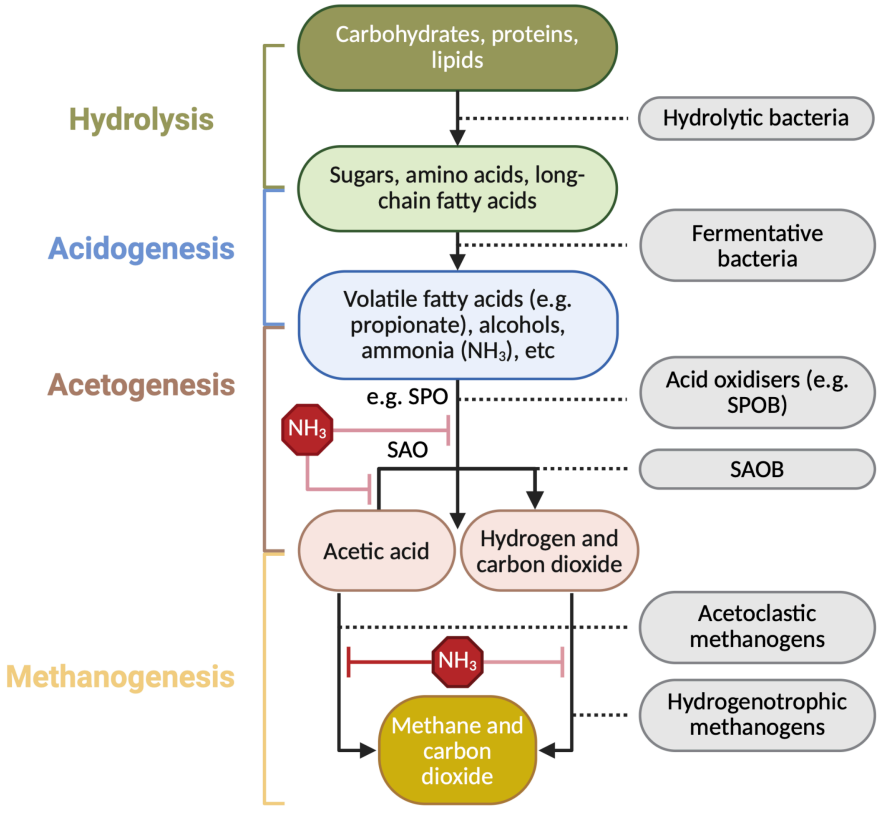

Figure 2. Simplified representation of the anaerobic digestion process and ammonia inhibition at its later steps.

During the initial stage (hydrolysis), microorganisms produce enzymes that degrade larger molecular compounds such as polysaccharides, lipids, and proteins into smaller molecules like sugars, long-chain fatty acids, and amino acids (Menzel et al. 2020). These are subsequently converted, in the next stage (acidogenesis), into VFA, such as propionic acid, acetic acid, or butyric acid, along with NH_3, H_2, and CO_2 (Sarker et al. 2019). In the third stage (acetogenesis), the previously produced acids with carbon chains longer than three carbon atoms are further degraded into acetate, H_2/formate, and CO_2 (Chen and Neibling 2014, Lohani and Havukainen 2018). In biogas production systems, acetate, H_2/formate and CO_2 are consumed in the final phase of the AD process (methanogenesis) to form CH_4 and CO_2, either by acetate-consuming (acetoclastic) or H_2/formate-consuming

(hydrogenotrophic) archaea (methanogens) (Patel et al. 2017, Schnürer and Jarvis 2018).

Due to the high diversity of microbial communities executing AD of organic wastes, both in terms of taxonomy and function, their presence and structure can be influenced and altered by operational factors such as substrate type, pH, temperature, and the presence of process inhibitors like NH_3 (Meegoda et al. 2018). Microbial communities have been shown to adapt to gradual and drastic changes in one or more of these operational factors, given that sufficient adaptation time is provided, for instance by operating at long HRT to avoid microbial wash-out. In these cases, AD can proceed even under conditions deemed toxic for certain microorganisms (Nakasaki et al. 2019, Buenaño-Vargas et al. 2024). An example of this is the operation under high NH_3 levels, where it has been demonstrated that microbial groups with key functions in the process are inhibited and replaced by ammonia-tolerant species that allow the continuation of the process (Yan et al. 2019, Puig-Castellví et al. 2020) (Figure 2).

2.2.1 Propionate and acetate accumulation, and the importance of syntrophic acid degradation in high-ammonia biogas reactors

The implications of NH_3-induced microbial shifts for industrial biogas production processes are highly relevant, as under these conditions, propionate and acetate can easily accumulate (Nielsen et al. 2007, Han et al. 2020, Yue et al. 2021). Propionate and acetate are important intermediary compounds in anaerobic digestion processes that can originate from the fermentation of several different substrates, such as lactate (Seeliger et al. 2002), carbohydrates (Crow 1988, Ueki et al. 2006) and amino acids (Barker 1981, Ramsay and Pullammanappallil 2001) or through the oxidation of long-chain fatty acids (Sousa et al. 2007). Moreover, acetate can also be formed by the syntrophic oxidation of ethanol (Du et al. 2024), propionate and butyrate (de Bok et al. 2001, Müller et al. 2010), and by the reduction of CO_2 through homoacetogenesis (Karekar et al. 2022). This plethora of pathways for the formation of propionate and acetate can lead to propionate and acetate accumulation, especially if an imbalance in the formation and consumption of these VFA is established. Acetate is mainly converted to methane by acetoclastic methanogens (AM) under low NH_3 levels (Li et al. 2015, Jiang et al. 2018). However, AM are notably vulnerable to NH_3 inhibition (Li et al. 2017, Wang et al. 2022), resulting in acetate buildup. In

such instances, ammonia-tolerant syntrophic microorganisms like SAOB and HM emerge as the primary consumers of acetate and H_2/formate and CO_2, respectively (Westerholm et al. 2016, Dyksma et al. 2020). As for propionate, it is degraded by SPOB, regardless of NH_3 levels, but the dominant species has been suggested to differ at various NH_3 levels (see chapter 3.1). Both SPOB and SAOB are usually slow growers (Scholten and Conrad 2000, Jannat et al. 2021, Papers I, II, III, and IV), which means that under disturbed conditions such as NH_3 inhibition, the consumption of propionate and acetate can become lower than the production, resulting in their accumulation. Thus, to maintain a stable biogas operation in high-ammonia digesters, identifying and studying these microorganisms to find the parameters that support their activities is highly relevant.

2.2.2 Syntrophy and thermodynamic constraints

Syntrophy refers to the mutual interdependence of two or more metabolically distinct microorganisms. For these microorganisms to fulfil their metabolic processes, they must closely cooperate to circumvent the thermodynamic constraints imposed by the product inhibition of the oxidation reactions (Table 1) (Ishii et al. 2005, Westerholm et al. 2015, Westerholm et al. 2022, Singh et al. 2023). As an example of such an interdependence, a recent study has demonstrated the existence of a syntrophic relationship between a bacterium that catalyses the production of methanol from formate and a methylotrophic methanogen that converts methanol into CH_4, CO_2, and H_2O (Huang et al. 2025). In this microbial interaction, methanol inhibits the bacterium unless the methanogen efficiently removes it, while the methanogen can only produce methane if methanol is available (Huang et al. 2025). Also, both SPOB and SAOB are obligately dependent on a syntrophic interaction with HM. In syntrophic propionate oxidation, SPOB converts propionate to acetate, H_2, and CO_2 (Figure 3). The formed acetate is consumed by AM in low-ammonia conditions and via acetate oxidation by SAOB at high ammonia levels (Singh et al. 2023, Weng et al. 2024). In the acetate oxidation, SAOB converts acetate to H_2/formate and CO_2 (Figure 3).

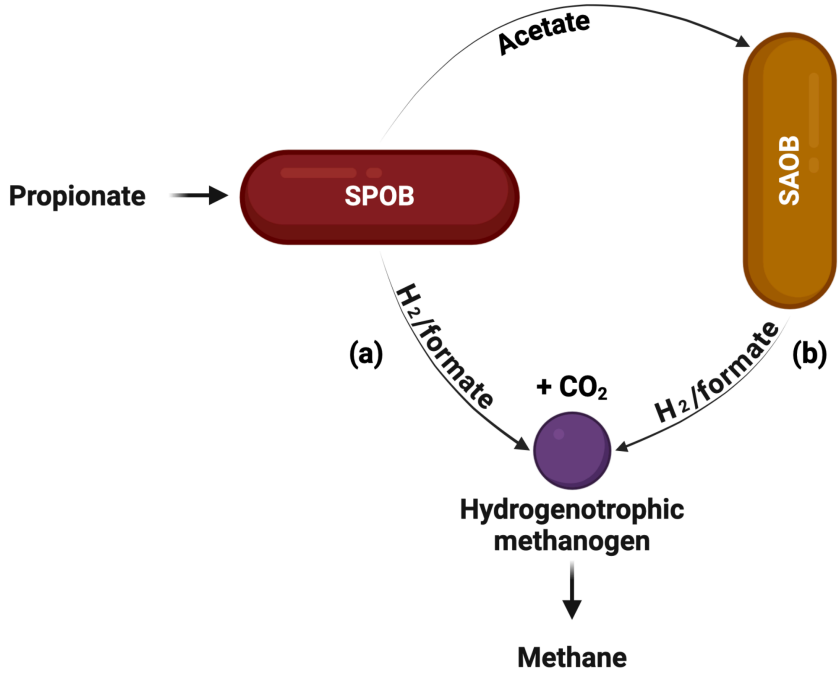

Figure 3. Schematic representation of syntrophic propionate consumption by syntrophic propionate-oxidising bacteria (SPOB) coupled with acetate consumption by syntrophic acetate-oxidising bacteria (SAOB) and the consumption of H_2 and/or formate by the hydrogenotrophic methanogen (HM), through the reduction of CO_2 into methane. Methanogenesis from propionate (a) and acetate (b) is shown.

However, if H_2/formate are not readily consumed by their partner HM, these oxidation reactions are not thermodynamically favourable (Weng et al. 2024, Paper I) (Table 1). This interdependence of catabolic reactions means that these microorganisms only gain energy if their metabolisms are coordinated and if H_2/formate partial pressures are kept at low values (Fukuzaki et al. 1990).

Table 1. Stoichiometric reactions and standard Gibbs free energy change ($\Delta G^{0'}$) of syntrophic propionate (through the MMC pathway) and acetate oxidation and hydrogenotrophic methanogenesis (Thauer et al. 1977). Methanogenesis from propionate (a) and acetate (b) is schematically shown in Figure 3.

Syntrophic metabolism	Reaction equation	$\Delta G^{0'}$ (kJ mol^{-1})
Propionate oxidation	(1) $CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2$	+76.1
Acetate oxidation	(2) $CH_3COOH + 2H_2O \rightarrow 2CO_2 + 4H_2$	+104.6
Hydrogenotrophic methanogenesis	(3) $2CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-135.6
Methanogenesis from propionate (a)	(1) + (3)	-59.5
Methanogenesis from acetate (b)	(2) + (3)	-31

While interspecies electron transfer (IET) often happens through the sharing of intermediary compounds like the abovementioned methanol (in the case of the methanol-producing bacterium and methylotrophic methanogen) and H_2/formate (in the case of SPOB, SAOB and HM) (Figure 4a), some syntrophic microorganisms (mostly belonging to the genus *Geobacter*) are capable of transferring electrons through direct interspecies electron transfer (DIET) (Summers et al. 2010, Rotaru et al. 2014) (Figure 4b and c).

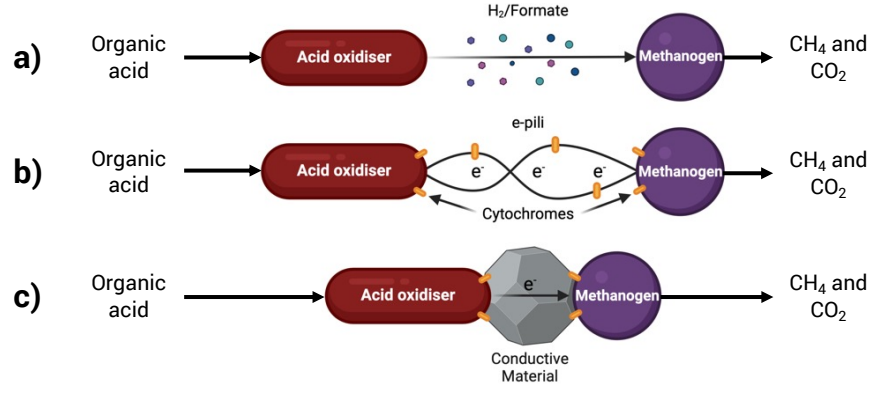

Figure 4. The different types of interspecies electron transfer (IET) between acid oxidisers and methanogens. IET can occur by transfer of H_2 or formate (a), of electrons through e-pili (b) or through direct contact with conductive materials (c).

DIET can happen via direct microbial contact through c-type cytochromes (Nevin and Lovley 2000, Holmes et al. 2021) and/or conductive pili (e-pili) (Reguera et al. 2005) (Figure 4b), or indirectly via contact with conductive materials (CM) (Lovley 2017) (Figure 4c) such as granular activated carbon (Liu et al. 2012), biochar (Chen et al. 2014) or iron oxides (Liu et al. 2015). This electron-allocation strategy gives syntrophic microorganisms another channel for electron transfer and could help overcome thermodynamic barriers, as it provides a slight advantage compared to IET through intermediary compounds (Storck et al. 2015). However, DIET has not been unequivocally established for SPOB and SAOB (except for the SPOB *Pelotomaculum thermopropionicum*, which has been suggested to perform DIET through conductive nanowires (e-pili) (Gorby et al. 2006)).

The syntrophic enrichment cultures used in this thesis's work have been derived from high-ammonia biogas processes (Westerholm et al. 2010, Singh et al. 2023) and were shown to be predominantly composed of syntrophic microorganisms (Papers I and IV). Investigating these enrichment cultures deepens our understanding of syntrophic microorganisms, enabling us to optimise their acid-degrading activity for biomethane production or regulate their metabolisms in other biotechnological processes, such as VFA or syngas production systems (Grimalt-Alemany et al. 2018, Liu et al. 2021).

3. Syntrophic propionate oxidation under high-ammonia conditions

3.1 Syntrophic propionate oxidisers (SPOB)

SPOB are found in the phylum Bacillota (previously Firmicutes), in the genera *Desulfofundulus* (Plugge et al. 2002, Watanabe et al. 2018) and *Pelotomaculum* (de Bok et al. 2005, Imachi et al. 2007, Hidalgo-Ahumada et al. 2018), and in the phylum Deltaproteobacteria, within the genera *Smithella* (de Bok et al. 2001), *Syntrophobacter* (Liu et al. 1999), and *Syntrophobacterium* (Wallrabenstein et al. 1995, Chen et al. 2005, Plugge et al. 2012). However, some yet-to-be-characterised SPOB are thought to belong to other bacterial taxa, including the uncultured phylum '*Candidatus* Cloacimonetes' (Pelletier et al. 2008, Dyksma and Gallert 2019) and members of the family Pelotomaculaceae (Hao et al. 2020, Singh et al. 2021, Singh et al. 2023, Papers I and IV). Certain SPOB are more susceptible to ammonia inhibition and are consequently often found in low ammonia conditions, such as those belonging to the genera *Smithella* (Zhang et al. 2018), *Syntrophobacter* (Sitthi et al. 2020), *Syntrophobacterium* (Bonk et al. 2018) and *Pelotomaculum* (Ban et al. 2013). Ammonia-tolerant SPOB are yet to be characterised, but metagenomic and transcriptomic studies of highly enriched propionate-degrading communities derived from high-ammonia biogas digesters have identified two SPOB candidates. They include the mesophilic '*Candidatus* Syntrophopropionicum ammoniitolerans' (Singh et al. 2021, Paper I) and the thermophilic '*Candidatus* Thermosyntrophopropionicum ammoniitolerans' (Singh et al. 2023, Paper IV), which belong to the family Pelotomaculaceae (Singh et al. 2021, Weng et al. 2024, Papers I and IV).

In pure culture, many of these species can ferment various substrates, including pyruvate and fumarate and use compounds such as sulfate and sulfite as electron acceptors when these compounds are present in the media (Harmsen et al. 1998, Chen et al. 2005). However, two members of the genus *Pelotomaculum*, namely *Pelotomaculum schinkii* (de Bok et al. 2005) and *Pelotomaculum propionicicum* (Imachi et al. 2007), have also been reported to be obligate propionate-oxidising syntrophs. In co-culture with a methanogen and in the presence of propionate, two pathways have been experimentally verified, with most of the known SPOB today degrading propionate via the methylmalonyl-CoA (MMC) pathway (Koch et al. 1983, Kosaka et al. 2006, Stams and Plugge 2009, Müller et al. 2010, Hidalgo-Ahumada et al. 2018, Sedano-Nunez et al. 2018) (Figure 5a).

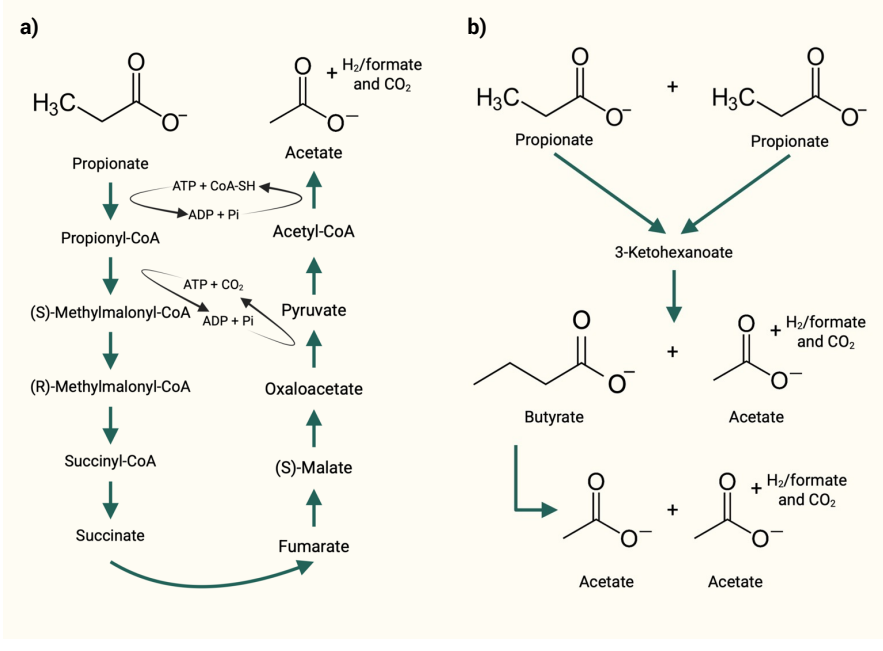

Figure 5. Simplified schematic representations of the MMC (a) and dismutating (b) pathways. Pathways modified from Singh et al. (2023) and de Bok et al. (2001).

Concordantly, the SPOB studied in this thesis ('*Ca.* S. ammoniitolerans' and '*Ca.* T. ammoniitolerans') have been shown to encode and express the genes involved in the MMC pathway (Singh et al. 2023, Weng et al. 2024). This pathway is characterised by an ATP-requiring initial step, which activates

propionate via a propionyl-CoA transferase and an ADP-forming ligase, coupled to the ATP-generating end reactions of the pathway (Kosaka et al. 2006, Kato et al. 2009) (Figure 5a). Furthermore, this propionate activation has also been suggested to occur autonomously through a CoA ligase (Hao et al. 2020). In the MMC pathway, propionate is enzymatically converted into succinate, which is subsequently oxidised to fumarate by a membrane-bound succinate dehydrogenase/fumarate reductase complex in the most energetically unfavourable reaction of the pathway. The resulting fumarate is then converted into pyruvate, generating ATP, before being further transformed into acetate (Singh et al. 2023) (Figure 5a). Both H_2/formate and CO_2 are produced during the process. The dismutating pathway employed by the genus *Smithella* has been proposed to be more energy-efficient than the MMC pathway (de Bok et al. 2001). Although both pathways require propionate activation, the dismutating pathway oxidises one propionate molecule into acetate, H_2/formate and CO_2, and reduces the other into butyrate (using H_2) (Figure 5b). The resulting butyrate is then degraded into two additional acetate molecules, H_2/formate and CO_2 (Liu et al. 1999, de Bok et al. 2001) (Figure 5b). Both propionate degradation pathways are energetically unfavourable unless their products (formate or H_2) are readily consumed. However, as the dismutating pathway generates less H_2, SPOB that possess this metabolic pathway are less thermodynamically constrained by H_2 than those that employ the MMC pathway (Dolfing 2013). Yet, in the context of SPO for biogas production, both require cooperation with different microorganisms (Stams 1994).

3.2 Cooperating microorganisms

3.2.1 Acetoclastic methanogens (AM) and syntrophic acetate-oxidising bacteria (SAOB)

Under low ammonia conditions, not only are the SPOB species different from those under high ammonia conditions, but they also have different acetate consumers as cooperating partners, with AM being the dominant acetate consumers under these conditions (Jiang et al. 2018). The known AM belong to the genera *Methanotrix* and *Methanosarcina*, both belonging to the order Methanosarcinales (Stams et al. 2019). While both of these genera are capable of utilising acetate to produce CH_4 and CO_2, *Methanosarcina* is

usually the dominant acetate consumer when acetate is present in higher concentrations (> 1 mM) and *Methanotrix* is the dominant acetotrophic genus under lower acetate concentrations (< 0.2 mM) (Zinder 1993). This happens because *Methanosarcina* has a higher maximum growth rate but a lower substrate affinity when compared with *Methanotrix*, meaning that it thrives when there is plenty of acetate available, while *Methanotrix*, with its higher substrate affinity but lower maximum growth rate, thrives at lower acetate concentrations (Zinder 1993). Acetoclastic methanogenesis is the dominant acetate consumption pathway under non-inhibited, mesophilic conditions, as the thermodynamics of this reaction generate more energy than SAO (Dyksma et al. 2020). However, AM are also often the most sensitive acetate consumers to ammonia inhibition (Fotidis et al. 2013, Fotidis et al. 2014) and, therefore, under high ammonia conditions, SAOB outcompete AM and become the main acetate degraders. Accordingly, in the highly enriched mesophilic and thermophilic syntrophic cultures (Singh et al. 2021, Singh et al. 2023) in the present study, SAOB were the only acetate degraders, whereas AM could not be detected (Papers I and IV). Like SPOB, most SAOB are classified under the phylum Bacillota. The majority of these organisms belong to the class Clostridia, like the mesophilic *Schnuerera ultunense* (Schnürer et al. 1996), *Tepidanaerobacter acetatoxydans* (Westerholm et al. 2011), the thermophilic *Thermacetogenium phaeum* (Hattori et al. 2000) and the mesophilic SAOB discussed in this thesis, *Syntrophaceticus schinkii*, and a thermophilic SAOB yet to be cultivated (Singh et al. 2023, Papers I and IV). Furthermore, a known thermophilic SAOB belonging to the phylum Thermotogae also exists (Balk et al. 2002). In cooperation with HM, these microorganisms degrade acetate through the reverse Wood-Ljungdahl pathway, producing H_2/formate and CO_2 (Hattori 2008).

3.2.2 Hydrogenotrophic methanogens (HM) commonly cooperating with syntrophic bacteria

In both low and high ammonia biogas processes, HM are essential for the steady consumption of H_2/formate that will allow both SPO and SAO to proceed (Stams 1994). HM are divided into two different phyla: Euryarchaeota and Crenarchaeota. However, the majority of known HM are found in the phylum Euryarchaeota, in genera such as *Methanobacterium*, *Methanothermobacter*, *Methanococcus*, *Methanomicrobium*,

Methanospirillum, and *Methanoculleus* (Enzmann et al. 2018). Some of these microorganisms can consume both formate and H_2 to produce CH_4 and CO_2 (Weng et al. 2024), but the preference for either molecule as an electron carrier is still debated (Thiele and Zeikus 1988, Hidalgo-Ahumada et al. 2018, Chen et al. 2020). Moreover, not all HM possess the genes coding for formate dehydrogenase, the enzyme that catalyses formate metabolism (Wagner et al. 2018). For these HM, this means that regardless of whether formate is produced, they can only produce CH_4 through the consumption of H_2 (Stams and Dong 1995, Worm et al. 2014). However, the HM typically involved in SPO and SAO have been shown to possess the genetic information enabling the use of both formate and H_2 (Hidalgo-Ahumada et al. 2018, Chen et al. 2020, Singh et al. 2023, Weng et al. 2024).

In the work presented in this thesis, the HM present in the mesophilic enrichment culture were identified as a novel species belonging to the genus *Methanoculleus*, specifically '*Candidatus* Methanoculleus ammoniitolerans' (Weng et al. 2024, Paper I). In the thermophilic enrichment culture, blast searches of the representative 16S rRNA gene sequence obtained in Paper IV indicated the presence of *Methanothermobacter tenebrarum* (Nakamura et al. 2013). This is a thermophilic methanogen isolated from a well in a natural gas field and also found as the main HM in a thermophilic fixed-bed anaerobic digester degrading organic wastes (Nagoya et al. 2020). Like other methanogens involved in SPO and SAO, the versatility to use both formate and H_2 as electron donors has also been shown to be present in *Ca*. M. ammoniitolerans (Weng et al. 2024) but not in *M. tenebrarum*, which grows exclusively on H_2/CO_2, like many species of this genus (Nakamura et al. 2013). These are interesting results since both the thermophilic SPOB '*Ca.* T. ammoniitolerans' and the novel thermophilic SAOB present in the cultures described in Paper IV were indicated to produce formate in a metatranscriptomic study of the thermophilic enrichment culture (Singh 2023). However, further molecular analyses of the genome of this *Methanothermobacter* are needed to confirm the identity of this species. Moreover, previous studies of the thermophilic enrichment culture also revealed the presence of a novel *Methanoculleus* (Singh et al. 2023). Species belonging to the genus *Methanoculleus*, like the HM *Methanoculleus bourgensis*, which is a common syntrophic partner to mesophilic SAOB at high ammonia conditions (Westerholm et al. 2016), have been shown to have H_2 thresholds as low as 0.1 Pa (i.e. high H_2 affinity) (Neubeck et al. 2016).

However, the '*Ca.* M. ammoniitolerans' present in the mesophilic enrichment culture used in the work present in this thesis, were shown to maintain the *p*H2 between 1.2-11 Pa and 0.6-14 Pa during SPO and SAO enrichment cultures (Weng et al. 2024, Paper I). As for *Methanothermobacter*, there is no available data on its H_2 threshold, nor for other species of the same genus. Yet, methanogens with low H_2 and/or formate thresholds could potentially be outcompeted by other HM, acetogens or formate-utilising bacteria at high H_2 or formate levels due to a low growth rate (Lee and Zinder 1988, Weijma et al. 2002, Laura and Jo 2023, Yu et al. 2023). It is well-known that the methanogenic community structure in a biogas production process is determined by several parameters, including substrate, pH, temperature of the process, affinities for formate and H_2, their threshold concentrations, and their capacity to use one or both compounds efficiently (Zinder 1993, Stams 1994, Chen et al. 2020). Hence, in processes dependent on syntrophic acid degradation, it is important to support the methanogenic species that can cooperate with SPOB and SAOB.

3.2.3 Importance of cell proximity between syntrophic cooperating species

Research is needed to look into what features are needed for the establishment of syntrophic interactions. It has been hypothesised that syntrophic relationships are formed between microbes that communicate either via pili/flagella-mediated interactions or chemical signalling mechanisms (Morris et al. 2013). For instance, a flagellum-mediated communication system was demonstrated between a syntrophic bacterium and a methanogen, where the bacterial flagellum ensured proximity between the cells and synchronised their metabolism (Shimoyama et al. 2009). Independent of the mechanisms used for interspecies cooperation, physical proximity between them is essential to efficiently exchange reaction products and potentially to communicate with each other (Thiele and Zeikus 1988, Ishii et al. 2005). This has been previously demonstrated for SPO cultures, where cell proximity was shown to be essential for effective H_2 transfer in between the SPOB and the partner HM (Ishii et al. 2005, Felchner-Zwirello et al. 2013). Concordantly, both the mesophilic SPO and SAO enrichment cultures formed large flocs during batch cultivations (Weng et al. 2024, Paper I). In contrast, the thermophilic enrichment culture did not form visible flocs similar to the mesophilic culture, even though small aggregates were

observed (Figure 6). The importance of close cell interactions was particularly emphasised for the mesophilic ammonia-tolerant '*Ca.* S. ammoniitolerans' in a recent study, where harsh stress caused by magnetic stirring critically disrupted the initial syntrophic connection between SPOB and methanogens, whereas the SAOB *S. schinkii* exhibited greater tolerance to shear stress and disruptive conditions that inhibited aggregate formation (Weng et al. 2025). This indicates that strategies to bring the SPOB and the cooperating HM closer can be particularly effective at high ammonia levels.

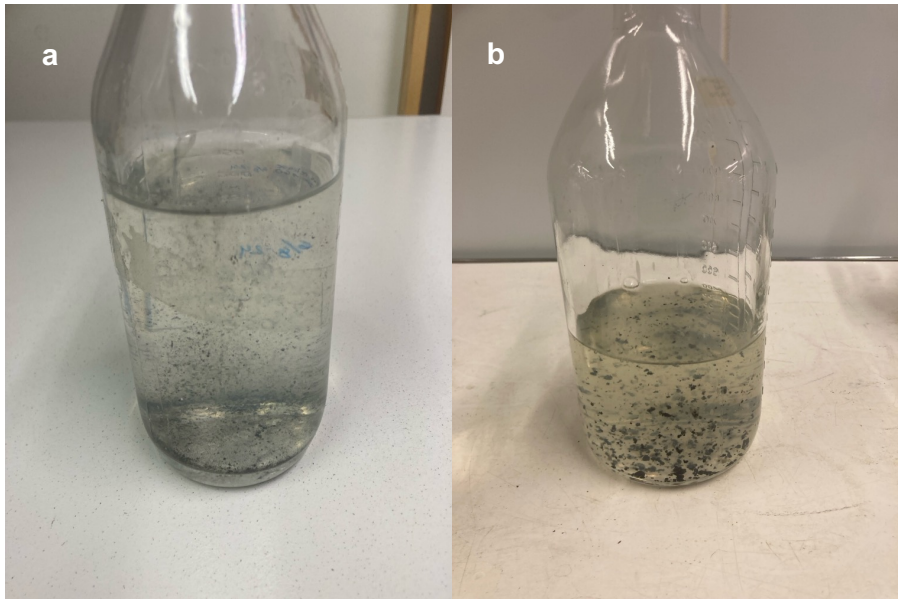

Figure 6. The difference between microbial floc size in thermophilic (a) and mesophilic (b) syntrophic propionate-oxidising (SPO) cultures.

4. Parameters impacting syntrophic metabolism under high ammonia conditions and potential implications on VFA accumulation in biogas production processes

4.1 Impact of starvation on SPOB and SAOB, and ammonia levels in SPOB

The intricacies of the metabolisms of ammonia-tolerant, syntrophic acid-oxidising bacteria can have different physiological implications, such as longer lag phases, slow growth and low metabolic rates, resulting in their typically low relative abundances in biogas production processes (Stams et al. 2012, Weng et al. 2024). In Paper I, where the effect of the inclusion of different additives in the SPO and SAO of mesophilic, ammonia-tolerant, syntrophic enrichment cultures was studied, these aspects of syntrophic microbial growth were likely exacerbated by starving both SPO and SAO cultures before feeding them with their respective substrates. However, the impact of starvation in SAOB was more pronounced, as it displayed much longer lag phases (49-56 days) than SPOB (28 days) after an initial substrate feeding (Paper I). Accordingly, after a second feeding of the respective substrates, the lag phase of SAOB was shortened to only 8 days, whereas SPOB's lag phase remained at between 22-36 days (Paper I). In this study, the propionate degradation and acetate degradation rates of both cultures had distinct changes between the first and second feedings with their respective substrates. While all SPO cultures increased their propionate degradation rates from 0.037-0.048 g L^{-1} day^{-1} to 0.095-0.130 g L^{-1} day^{-1}, one of the

treatments (graphene, 0.067 vs 0.062 g L^{-1} day^{-1}) and, more importantly, the controls (0.083 vs 0.081 g L^{-1} day^{-1}) of the SAO cultures did not improve their acetate degradation rates (Paper I). This demonstrates that while both cultures displayed noticeably greater biomass during the second round of acid degradation, and that key metabolic enzymes may have already been present during the second feeding (Brock et al. 2003, Paper I), only the SPO culture's acid degradation capacity seems to have benefited from this. Nevertheless, the fact that the SPO's culture lag phase did not improve between feedings, but the lag phase of the SAO culture did, could also indicate physiological benefits from increased biomass for SAOB.

In Paper II, the effect of different NH_3 levels on the lag phase and propionate degradation rates of mesophilic SPOB was clear, with concentrations of 0.1 M, 0.2 M, and 0.3 M of NH_4Cl yielding lag phases of 28, 41, and 41 days and propionate degradation rates of 0.588, 0.267 and 0.213 mmol day^{-1}, respectively; the highest concentration tested (0.5 M of NH_4Cl) prolonged the lag phase for over 150 days (Paper II) and thus had a propionate degradation rate of 0.065 mmol day^{-1} (Paper II). According to these results, the prediction models employed in Paper II determined that increasing NH_3 concentrations are expected to worsen both the lag phases and the propionate degradation rates of SPO cultures. In Paper III, the effect of different NH_3 concentrations was even more evident, as trying to grow thermophilic SPO cultures at 0.3 M of NH_4Cl did not yield significant propionate degradation. However, based on the propionate degradation rates of the cultures employed in this Paper, the prediction model forecasted that the highest propionate degradation rates (of around 0.05 mmol day^{-1}) could be achieved even at 0.2 M of NH_4Cl (Paper III).

The long lag phases and slow acid degradation rates of SPOB and SAOB have been reported in other studies using the same enrichment cultures as in Paper I (Weng et al. 2024, Weng et al. 2025), and in other syntrophic microorganisms (Westerholm et al. 2019). While these lag phases are not likely to be the case in an industrial biogas reactor that is constantly fed substrate and in which intermediates such as propionate and acetate are consistently formed, in these complex communities, there is a constant competition for substrates between various microbial groups. For instance, under low-ammonia conditions, the ammonia-tolerant syntrophic species might not be able to compete for the substrate with more efficient SPOB or AM. Hence, these species can enter a starvation mode and become low in

abundance. In such a case, the low activity exhibited by these microorganisms requires a higher HRT and lower OLR for their establishment and growth, and to avoid their wash-out of the process. This is particularly important for biogas production processes that are prone to experiencing events of prolonged NH_3 inhibition and consequent propionate and acetate accumulation. However, once these microorganisms are well-established and active (i.e. at a steady-state), both propionate and acetate can be steadily consumed. Altogether, these results underscore the importance of considering the biological specificities of the ammonia-tolerant species, such as long lag phases, to account for the event of rapid NH_3 inhibition in biogas processes.

4.2 Impact of temperature and pH in SPOB

Temperature is an important factor determining growth and degradation rates, since raising the temperature (within microbial growth limits) is expected to enhance the thermodynamic favourability and kinetics of SPO and SAO and hydrogenotrophic methanogenesis. Accordingly, research indicates that mesophilic SPOB, SAOB and HM benefit from higher temperatures (Fey and Conrad 2000, Gan et al. 2012, Westerholm et al. 2019). However, interestingly, a similar trend was observed in the mesophilic SPO culture amended with zeolites only within a very narrow temperature range (Paper II). While this SPO enrichment culture benefited from an increase in temperature from 35-38.5°C, as shown by the positive impacts predicted in its different performance metrics, such as lag phase, propionate degradation rate and, especially, methane production rate, this SPO enrichment amended with zeolites could only grow within this narrow temperature span, as attempts to cultivate it at 40°C did not succeed. However, it is noteworthy to point out that screening experiments for the setup of the main experiment described in Paper II demonstrated that this enrichment culture could not only grow at 40°C, but also had better propionate degradation performance than when grown at 37°C. The reason for the failure of its growth at 40°C when amended with zeolites is thus, enigmatic.

As mesophilic syntrophic microorganisms often have better performance metrics at higher temperatures, thermophilic syntrophic microorganisms often have better performance metrics than their mesophilic counterparts

(Moset et al. 2015, Zhao et al. 2018). However, this was not the case for any of the propionate-oxidising cultures contained in this thesis (Papers III and IV). While the mesophilic SPO culture in Paper II demonstrated propionate degradation rates between 0.25-0.55 mmol day^{-1}, the thermophilic SPO culture in Paper III had propionate degradation rates ten times lower, between 0.025-0.050 mmol day^{-1}. Moreover, the controls of these same enrichment cultures used in Paper IV showed a similar scenario, with mesophilic SPO enrichment cultures showing a propionate degradation rate of 0.204 mmol day^{-1} while their thermophilic counterparts had a propionate degradation rate of 0.030 mmol day^{-1}. In this same Paper, the capacity of sludges originating from mesophilic and thermophilic industrial biogas processes to degrade propionate was also assessed. Once again, propionate was degraded faster in the mesophilic sludge than in the thermophilic sludge, with propionate degradation rates of 9.36 mmol day^{-1} and 4.74 mmol day^{-1}, respectively. These slower propionate degradation rates then resulted in lower methane production rates (Paper IV). While the reason for this difference in propionate degradation rate between mesophilic and thermophilic propionate-oxidising cultures is not clear, some studies have suggested that this could be related to higher NH_3 stress in thermophilic conditions (Singh et al. 2023), lower microbial diversity (Levén et al. 2007) and propionate toxicity (Speece et al. 2006). Hence, the process's temperature is an important parameter to consider in industrial-scale plants that operate at high ammonia and depend on the ammonia-tolerant SPOB.

Similar to temperature, the significance of pH in regulating the NH_3 fraction in the process is of great importance and has substantial implications for process performance (Rajagopal et al. 2013). This observation has also been noted in Papers I and III, where elevated pH levels resulted in inhibitory concentrations of NH_3. It is noteworthy that in these studies, an increase in pH is detected upon the onset of acid degradation. This is most likely because, at that moment, methanogens begin consuming CO_2 (which, by reacting with H_2O, forms carbonic acid, thereby lowering the pH) and H_2, the low levels of which ultimately trigger a faster acid degradation. In the case of Paper I, a sudden increase in pH during the syntrophic degradation of propionate caused a corresponding rise in NH_3; although a direct correlation cannot be established, this coincided with a plateau phase of propionate degradation and a sharp increase in H_2 levels, possibly indicating NH_3 inhibition of SPOB and the partner HM, but also likely prompted by the

start of faster acetate consumption (Paper I). Following the degradation of the accumulated acetate, propionate degradation resumed, and the H_2 levels dropped significantly as soon as the pH decreased, along with a corresponding reduction in the NH_3 fraction.

4.3 Impact of propionate levels in SPOB

In addition to temperature, propionate levels are an important factor for SPO. According to Le Chatelier's Principle, increased substrate levels usually favour the thermodynamics of endergonic reactions like SPO by shifting their equilibrium towards product formation. Yet, high propionate levels at different set temperatures have been shown to inhibit methanogenesis (Barredo and Evison 1991, Li et al. 2020). Specifically, an increase in propionate concentration from 0.4 to 6.9 g L^{-1} under thermophilic conditions (55°C) has been noted to cause inhibition, while no such inhibition occurred under mesophilic conditions (35°C) (Li et al. 2020). Additionally, propionate has been reported to inhibit methanogenesis in mesophilic conditions (37°C) at concentrations as low as 20 mM, with stronger inhibition observed at 80 mM, though at different pH levels (Barredo and Evison 1991). In systems with restricted buffering capacity, the inhibition of high propionate levels can be linked to a decrease in the pH of the media, as under thermophilic conditions, the dissociation of propionic acid is promoted (Mu et al. 2018, Zhang et al. 2018). Thus, it is noteworthy that Barredo and Evison (1991) observed greater cumulative methane production by a methanogen-enriched sludge at pH 8 than at pH 6.5 or 7. These results are also described in the work of this thesis. While SPOB cultivation was possible at propionate concentrations of 50 mM (starting pH of 7.3, Papers I, II and IV), in mesophilic conditions (37°C) and with a medium buffer of N_2/CO_2 (which lowers pH levels), this was not feasible under thermophilic conditions (Paper IV). For this reason, the thermophilic SPOB could only be cultured at a starting propionate concentration of 30 mM (Papers III and IV), and by only using N_2 as a gas phase in order to increase pH levels.

4.4 Impact of acetate levels in SPOB

Additionally, in both Paper I and another study (Weng et al. 2024), plateaus in propionate degradation were observed alongside significant peaks in acetate, NH_3, and H_2 levels in the mesophilic enrichment culture. This plateau was most likely not caused by changes in microbial community structure, as the same methanogen was the cooperating partner for both SPOB and SAOB in this culture, as indicated by 16S rRNA gene sequencing and transcriptomic analyses (Paper I, Weng et al. 2024). Several studies (Gorris et al. 1989, Fukuzaki et al. 1990) have suggested a correlation between this halt in SPO and peaking acetate levels. Nevertheless, additions of up to 2 g L^{-1} of acetate have demonstrated no significant hindrance of propionate degradation at an initial concentration of 3.5 g L^{-1} (Paper I). Moreover, thermodynamic calculations from recent studies (Weng et al. 2024) indicate that this phenomenon is more likely associated with peaks in H_2 and NH_3 levels (caused by a temporary increase in pH), which aligns with the results obtained in Paper I. Indeed, this halt in SPO could involve the metabolic shifts in the community including variation in the production and consumption of H_2 and formate as intermediate compounds, or it may be related to temporary changes in redox potential.

5. Strategies to improve SPO metabolism under high ammonia conditions

5.1 Presence of macro- and micronutrients

Increased knowledge of the nutritional requirements for syntrophic acid degradation can help develop strategies to lower VFA accumulation at high ammonia levels. However, in addition to facilitating the SPO metabolism, an important part of the biogas production processes operating at high ammonia is to ensure that all nutrient requirements are met. It is well-known that the nutritional requirements of microorganisms change with environmental conditions. For instance, under high ammonia conditions, nutrients related to stress response mechanisms can be particularly important (Hendriks et al. 2017). Macronutrients such as potassium (K), phosphorus (P), sulfur (S), calcium (Ca), and magnesium (Mg) are vital for various basic metabolic functions involved in microbial growth, including cation pumps (K) (Takashima et al. 1990), nucleic acid functionality (P and Mg) (Inatomi 1986, Takashima et al. 1990), protein formation and functionality (S and Ca) (Bryant et al. 1971, Forsén and Kördel 1994), as well as cell aggregation (Das et al. 2014) and signalling (Ca) (Forsén and Kördel 1994). Conversely, micronutrients (also referred to in biogas production processes as trace elements) such as iron (Fe), selenium (Se), zinc (Zn), cobalt (Co), and nickel (Ni) serve as cofactors in enzymes responsible for energy production and cell maintenance, playing a particularly critical role in methanogenesis and especially in response to extreme conditions (Hendriks et al. 2017). While these nutrients are important for the metabolism of ammonia-tolerant syntrophic microorganisms, they could also potentially improve the ammonia tolerance and activity of species that compete for substrates used

by syntrophic bacteria. Accordingly, several studies have demonstrated that the addition of trace elements lowers VFA degradation and improves methane production in high-ammonia processes (Banks et al. 2012, Westerholm et al. 2015, Capson-Tojo et al. 2018). However, in Westerholm et al. (2015), the abundance of known SAOB was lower in digesters supplemented with trace elements, indicating that other species that degrade acetate more efficiently were able to compete with the SAOB.

Some materials, like zeolites, can promote the bioavailability of some of these trace elements. Zeolites are porous aluminosilicates commonly used in wastewater treatment (Kalló 2001) and as soil amendments (Eroglu et al. 2017) due to their ion exchange capacity, which allows for the removal of toxic compounds like metals and NH_4^+ from soils and water bodies in exchange for some of the naturally occurring ions (these vary depending on the type of zeolite) in the zeolite (Tsadilas et al. 1997, Wang and Peng 2010). In biogas production processes under high NH_3 conditions, this property of zeolites has been used to promote the absorption of NH_4^+ in exchange for the zeolites' surface cations (Ho and Ho 2012, Zheng et al. 2015), though not all zeolites have an equal capacity to remove NH_4^+ from the medium (Tada et al. 2005, Langwaldt 2008). Although it cannot be definitively stated that the addition of zeolites in the work contained in this thesis assuredly promoted SPO and SAO through the bioavailability of chemical species like Ca and Mg, it is reasonable to theorise that, given their ion exchange capacities, this was likely the case (Papers I, II, and III). Not only were Ca and Mg two of the most abundant ionic species present on the surface of the zeolites used (clinoptilolite; particle size 0.5-1 mm, Zeo-Concept Ece AB), but the roles these nutrients play are crucial for the proper functioning of microbial cells. The increased bioavailability of Ca may clarify why the addition of zeolites promoted the aggregation of syntrophic species in both the SPO and SAO cultures (Paper I). This, combined with the availability of additional Mg, may account for the improved acid-degrading performance in both the SPO and SAO cultures (Paper I), as aggregation reduces interspecies distances, which is crucial for overcoming the thermodynamic barriers of syntrophic metabolism by facilitating the exchange of products between bacteria and methanogens (de Bok et al. 2004, Ishii et al. 2005, Felchner-Zwirello et al. 2013). The presence of these two nutrients may also explain why higher dosages of these zeolites are particularly important for enhancing SPO at elevated NH_4^+ concentrations and temperatures in mesophilic regimes (Paper

II), as Mg and Ca are involved in nucleic acid functionality and protein formation and functionality, respectively - processes that are essential for maintaining metabolic activities, particularly under stressed conditions. Interestingly, however, no significant removal of NH_4^+ from the media was detected by applying these zeolites (Paper I).

5.2 Addition of Fe and S compounds

Other essential nutrients for microbial growth, though in different quantities, are iron (Fe) and sulfur (S) (Hendriks et al. 2017). At the cellular level, Fe is integral to protein function as a biocatalyst, in DNA biosynthesis and electron transfer. Furthermore, in SPO and methanogenesis, Fe, along with S, is vital for several metabolic pathway enzymes (Müller et al. 2010, Westerholm et al. 2022). In an AD process, Fe is frequently added to promote the precipitation of S (forming FeS and FeS_2) to help prevent the formation of H_2S. This compound is corrosive for the industrial equipment involved in biogas production processes and toxic for the microbial groups performing AD and methanogenesis, either by direct inhibition (Chen et al. 2008, Lopes et al. 2010, Tan et al. 2019, Vu et al. 2022) or by reacting with trace metals that are necessary as micronutrients (van der Veen et al. 2007, Chen et al. 2008). The formation of pyrites (FeS_2, cubic) or FeS is dependent on the conditions of the media (like pH) (Lyon 2010), and while it may make Fe less bioavailable, it also forms precipitates that can serve as support structures in anaerobic environments, as seen in the mesophilic enrichment cultures amended with iron oxides in Paper I. In this way, FeS_2 precipitate formation and the visible biofilm development in these precipitates could explain the improved propionate and acetate degradation rates in the iron oxide amended cultures of the mesophilic enrichment cultures' in Paper I. Adding Fe to the AD process also plays a role in trapping P through its reaction with iron oxides (Wei et al. 2018), which is significant, as excessive concentrations of P can lead to the inhibition of several microbial groups (Mancipe-Jiménez et al. 2017). Again, the results obtained on Paper I, in which the addition of iron oxide nanoparticles and zeolites improved both acetate and propionate degradation rates, and promoted an extensive formation of phosphate precipitates, particularly in the form of vivianite ($Fe_3^{2+}(PO_4)_2{\cdot}8H_2O$), provide further indications of the importance of phosphate precipitation in AD processes.

The multitude of cellular and operational functions of Fe, along with the cellular functions of S, makes their addition to biogas production processes a promising strategy for improving syntrophic metabolisms. In fact, while the addition of different Fe and S compounds yielded no improvements in mesophilic enrichment cultures, in the thermophilic syntrophic enrichment cultures, the addition of the chemical compounds $FeSO_4$ and $FeCl_2$ had a positive impact on both the SPO and the methane generation rate (Paper IV). This positive impact of both compounds may be related to the higher availability of Fe^{2+} in the media, which is crucial given the multitude of enzymes involved in fermentation, where Fe functions as a cofactor (Liu et al. 2012). However, in a replicate experimental setup employed to determine the impact of these two compounds on microbial community dynamics of the thermophilic enrichment culture, the positive impact of $FeCl_2$ in SPO was not observed again, for unknown reasons (Paper IV).

The complex chemical and microbiological factors that need to be considered during the addition of Fe and S compounds are further emphasised by the microbial community analyses of the thermophilic SPO enrichments in Paper IV. Here, both the addition of $FeCl_2$ and $FeSO_4$ resulted in a high abundance of a member of the genus *Acetomicrobium* alongside the SPO candidate. However, since the addition of $FeCl_2$ did not improve propionate degradation in this setup, this species is most likely not involved in SPO or SAO. While its activity in the culture is not clear, the ability of certain species belonging to this genus to use yeast extract for their growth may indicate that this member of *Acetomicrobium* uses the yeast extract present in the medium to grow (Winter et al. 1987, Hania et al. 2016).

5.2.1 Presence of sulfate and the potential combined effect of adding $FeSO_4$

Some SPOB, though not all (Liu et al. 1999, Imachi et al. 2002, de Bok et al. 2005, Imachi et al. 2007), are capable of reducing sulfate in its presence and are, therefore, phylogenetically closely related to another group of microorganisms capable of oxidising propionate, namely sulfate-reducing bacteria (SRB) (Watanabe et al. 2018, Singh et al. 2023). These microorganisms oxidise propionate and produce acetate and hydrogen sulfide (H_2S) through the reduction of sulfate (Moestedt et al. 2013). During this process, H_2 is produced, but it is kept at low concentrations as SRB use it as an electron donor for the reduction of sulfate to H_2S (Núñez 2018). For

SPOB that possess this metabolism, when sulfate (or compounds such as thiosulfates and sulfides) is present in the media, they use it as a preferential electron acceptor, as sulfate has a more positive standard reduction potential than H_2, meaning that more energy is generated when sulfate is reduced (Colleran et al. 1995, Hoehler et al. 2001). Moreover, some SRB are capable of consuming acetate, and others are capable of using H_2 at particularly low partial pressures, rendering them competitive with acetate oxidisers and HM (Stefanie et al. 1994, Stams et al. 2003, Liu et al. 2018). This implies that in biogas production processes where SAOB are the main acetate consumers and HM are the main methane producers, the presence of sulfate in the substrate prevents these microorganisms from accessing the substrates needed for their metabolisms, and methane yields can be severely affected (Madden et al. 2014, Lackner et al. 2020). Yet, adding low levels of sulfur species to biogas production processes can also help improve methane production rates by facilitating the initiation of propionate oxidation to acetate and the consequent methane production rate (Li et al. 2015, Zan and Hao 2020, Paper IV).

The significantly positive impact of $FeSO_4$ addition to the thermophilic enrichment cultures in Paper IV cannot be solely attributed to the increased abundance of both Fe^{2+} and SO_4^{2-} in the media (Paper IV). This is because the elevated levels of Fe^{2+} or SO_4^{2-} alone did not yield any positive results, as indicated by the $FeCl_2$ and Na_2SO_4 results, respectively (Paper IV). This is interesting since the thermophilic SPOB '*Ca.* T. ammoniitolerans, the only SPOB present in these enrichment cultures (Paper IV), possesses the genes for sulfate reduction. Furthermore, metatranscriptomic studies have suggested that this thermophilic SPOB and the HM partner *Methanothermobacter* utilise sulfuric compounds to exchange electrons (Singh et al. 2023). This has also been suggested for the thermophilic SPOB candidate 'Candidatus Propionivorax syntrophicum' (Hao et al. 2020, Singh et al. 2023). Hence, future studies should confirm if and, in that case, which sulfuric species the syntrophic microorganisms use in their cooperation. Furthermore, if SO_4^{2-} actually was used as an electron acceptor and facilitated the initiation of SPO metabolism, a lower propionate-to-methane conversion should have been registered. Interestingly, this was contradicted by carbon balance calculations performed in Paper IV, which showed a 105% methane yield based on the theoretical methane potential. Therefore, the reason for the positive effect of $FeSO_4$ addition has to be due to a more complex

mechanism. Elemental analyses of Fe and S in the cultivation media, formation of H_2S from sulfate reduction and metatranscriptomic analyses of the SPO enrichment culture are some of the analyses that could provide more insights into this.

The addition of $FeSO_4$ to ammonia-tolerant, syntrophic enrichment cultures adapted to a mesophilic regime did not produce faster propionate degradation or methane production rates (Paper IV). While this is in line with the lack of positive effects of the addition of other Fe and S compounds, it is noteworthy that the genes found in the thermophilic SPOB, indicating the capacity for sulfate reduction, were not present in the mesophilic SPOB (Paper IV).

5.3 Addition of materials

As referred to in sub-chapter 3.2.3, microbial aggregation is an important strategy to improve syntrophic metabolisms (de Bok et al. 2004, Ishii et al. 2005, Felchner-Zwirello et al. 2013). Aggregation of microorganisms seems to have developed both as protection against toxic compounds, but also has practical implications in syntrophic metabolisms as a means to share the products of their metabolisms effectively (Trego et al. 2021). It is possible that aggregation protects these organisms by reducing their contact surface with toxic compounds (while helping to maintain beneficial growth conditions within the core of the aggregate). In addition, aggregation also provides a possibility for IET through H_2/formate (Figure 4a) to occur more efficiently by shortening intercellular distances (Ishii et al. 2005, Stams and Plugge 2009, Doloman and Sousa 2024), aiding anaerobic processes in avoiding product inhibition and all its consequences in biogas production processes (Ishii et al. 2005, Felchner-Zwirello et al. 2013). This approach to enhancing IET has been demonstrated in various studies concerning different syntrophic microbial communities, including syntrophic butyrate-oxidising communities (Cong et al. 2021) and SPO communities (Kim et al. 2002, Ishii et al. 2005, Paper I). The metabolic advantages underlying aggregation in syntrophic anaerobic microorganisms are clear from their relative abundance in aggregates compared to their presence in the planktonic state (Doloman et al. 2024, Doloman and Sousa 2024, Paper I). While IET via formate and H_2 constitutes one of the most important and studied means of energy transfer,

syntrophic microorganisms can also transfer electrons through different DIET mechanisms (Figure 4b and c), as explained in section 2.2.2.

Numerous studies suggest that the capacity to exchange electrons through DIET requires close associations between microorganisms, whether directly through cell-to-cell interactions or indirectly via contact with CM (Lovley 2017) (Figure 4b and c). The potential stimulation of DIET between syntrophic species through the addition of CM has led to various research studies where carbon-based and metallic CM are incorporated into biogas production processes degrading complex substrates under high ammonia conditions, where syntrophic microorganisms are particularly critical for process stability (Abdelsalam et al. 2017, Johnravindar et al. 2020, Rasapoor et al. 2020, Yun et al. 2021, Li et al. 2022, Singh et al. 2022, Kalantzis et al. 2023). All these studies report improved VFA degradation and methane generation in their complex cultures, showing the suitability of adding these materials to improve biogas production processes, while attributing these results to DIET. However, none of these studies were conducted with highly enriched syntrophic cultures, nor have they clearly demonstrated DIET occurrence between syntrophic microorganisms. In the work presented in this thesis, we assessed the impact of some of the most extensively researched carbon and metallic CM, namely graphene and iron oxide (II, III) nanoparticles, on the degradation of VFA propionate and acetate and the corresponding methane generation by highly enriched SPO and SAO cultures (Paper I). The addition of iron oxide (II, III) nanoparticles promoted both the propionate degradation rate and acetate degradation, although only after a second substrate feeding, whereas the addition of graphene facilitated only propionate degradation (Paper I). Concurrently, the quantitative abundance of the main SPOB in these cultures was found to be higher in the aggregates (flocks) of the iron oxide-amended cultures compared to the control. As for the graphene-amended cultures, syntrophic species were only found in higher numbers than in the control in aggregates of the acetate-degrading communities, and these differences were not significant (Paper I). Furthermore, scanning electron microscopy (SEM) images of selected samples from these cultures allowed us to observe that the iron oxide-amended cultures exhibited substantial biofilm formation associated with the development of Fe precipitates (pyrites), whereas the graphene-amended cultures did not show extensive biofilm formation around the graphene nanoparticles, which might reflect the less significant improvement in VFA

degradation within these cultures (Paper I). While the addition of graphene does not appear to have yielded the same promising results as in other studies (Lin et al. 2018, Liu et al. 2020, Yadav et al. 2024), this may be attributed to the structure of the graphene used in Paper I. From the SEM images of the graphene cultures, it is evident that the conformation of the carbon sheets, with a lack of suitable pockets for microbial interactions, does not seem optimal for the biofilm formation necessary for the metabolisms of syntrophic species. Moreover, this conformation of graphene has been shown in other studies to have antibacterial activities by means of cell membrane damage (Akhavan and Ghaderi 2010) and oxidative stress (Gurunathan et al. 2012). In contrast, the structure of other electrically conductive carbon materials, such as granular activated carbon, does offer physical spaces for microorganisms to establish connections (Florentino et al. 2019), highlighting the significance of not only the materials' conductivity but also their physical structure. Although not directly demonstrating that these microorganisms possess the capacity to perform DIET, the combined improved acid degradation and recruitment of syntrophic species in flocks of CM-amended cultures further suggests this scenario (Paper I). This opportunity provides another pathway for syntrophic organisms to exchange electrons between species, potentially benefiting the AD process (Viggi et al. 2014).

Another strategy to improve syntrophic metabolism is the addition of carrier materials that, although not conductive, can still promote microbial aggregation, such as zeolites. While some of the benefits of zeolite addition have already been addressed in section 5.1, zeolites can also promote syntrophic metabolism as their porosity promotes microbial aggregation (Hrenovic et al. 2003, Tang et al. 2023, Paper I), which would theoretically enhance IET (through H_2 and formate) and DIET. Based on this and on zeolite's capacity to remove ammonia from solutions, several studies have investigated the impact of their addition to biogas production processes degrading complex substrates under high ammonia conditions. These studies reported positive outcomes such as increased maximum cumulative methane production and removal efficiencies in total chemical oxygen demand (Milán et al. 2001, Montalvo et al. 2005, Tada et al. 2005). These results are in accordance with the results of Paper I, where in mesophilic SPO and SAO, zeolites were the material that exhibited the best acid degradation rates following a second feeding of the culture's acid (Paper I). Furthermore, the

results demonstrated a greater abundance of syntrophic species in the flocs (with the exception of the main SAOB in the propionate-degrading community) than in the planktonic community. Additionally, through the SEM images, it was possible to discern extensive biofilm formation around different types of phosphate precipitates in these cultures, along with the porous nature of these precipitates, which may have facilitated the syntrophic metabolism and further points to the significance of the materials' physical structure in supporting syntrophic metabolisms (Paper I). While zeolites are often employed to remove ammonia from the medium, measurements taken in that experiment revealed no significant differences in ammonia (or ammonium) levels between the zeolite-treated and control cultures, suggesting that the benefits provided by the addition of zeolites may have been more related to the formation of the precipitates and the creation of spaces for the syntrophic species to establish connections amongst themselves, thereby aiding their metabolisms. This indicates that, regardless of whether SPOB and SAOB are capable of DIET, zeolites most likely facilitate IET through formate and H_2. The extent to which zeolites could contribute to other biogas production processes was addressed in two additional Papers contained in this thesis (Papers II and III), where the synergistic effects between their dosages, temperatures (across different temperature regimes), and ammonia levels were assessed. While their addition was not predicted to yield significant improvements in mesophilic SPO, it was predicted to have a considerable impact on the lag phase of the cultures, both in acetate accumulation and degradation, as well as in the rate of methanogenesis, with a particularly positive impact at higher ammonia concentrations (Papers II). As for the impacts of zeolites in the thermophilic cultures, their addition at dosages between 4-8 g L^{-1} across the tested temperatures of 50-54°C was predicted to yield the highest possible propionate degradation rate, although ammonium levels slightly impacted the optimal zeolite dosage (Paper III). These results demonstrate great potential for the addition of these materials to enhance syntrophic microbial growth and biogas production in processes under high ammonia conditions.

5.4 Industrial applications

The application of process parameters that take into account the biological specificities of the syntrophic microorganisms thriving in anaerobic digesters

treating different substrates is an optimisation strategy that can and should be applied to all biogas production processes. While there are several ways to mitigate ammonia inhibition, it is also possible to achieve good results while operating at high ammonia conditions if the occurrence of high ammonia levels is well predicted and measures to address that are taken. For instance, to start a biogas production process treating substrates likely to generate a high level of ammonia, using acclimated cultures has been shown to promote the effective AD of these substrates (Tian et al. 2018). Moreover, non-acclimated cultures can still thrive and maintain a stable biogas production performance if the introduction of the disturbance factor is done progressively and the specificities of the resulting microbial community are taken into account (Puig-Castellví et al. 2020).

The addition of both chemical compounds and different types of materials for the improvement of biogas production by syntrophic cultures under high ammonia conditions was demonstrated in this thesis. Although most of the experiments conducted in this thesis were not conducted in industrial-scale reactors nor in reactors with the specificities that industrial-scale reactors have, some of the optimisation strategies proposed here have significant impacts on the anaerobic oxidation under high ammonia conditions of crucial intermediary compounds like propionate and acetate that often generate problems in industrial settings if accumulated. Moreover, the possibility of performing these studies using highly enriched, ammonia-tolerant, syntrophic SPO and SAO communities is a novelty that allows for studying complex interactions in a group of microorganisms that not only is extremely challenging to study due to their interdependent metabolisms but is also of great importance for maintaining a stable biogas production operation under high ammonia conditions. While there are many studies on the influences of the addition of these materials and nutrients in complex cultures (Liu et al. 2020, Zhang et al. 2020, Singh et al. 2022, Tang et al. 2023), understanding how optimisation strategies specifically impact these cultures by promoting propionate and acetate degradation, and methane production is valuable knowledge. While there are limitations in extrapolating the results described here to industrial biogas processes, these results allow us to understand an essential piece of propionate and acetate oxidation in biogas processes operating under high ammonia conditions. Some of the additives proposed in this thesis, such as iron chloride, are already commonly employed in biogas production processes (as described in chapter 5.2) for different

operational reasons (Lin et al. 2017), while others are not and may have beneficial or undesired consequences for the structural integrity of the process (plumbing, etc.) and/or for the microbiological aspect of it. While the impact of the additives used in this thesis was studied in syntrophic enrichment cultures, their impacts may differ in industrial biogas production processes, depending on reactor configuration, the type of substrate being degraded, the process's temperature, if the process is experiencing some kind of inhibition, and, if so, which kind of inhibition. Furthermore, competition between various trophic microbial groups for substrate and nutrients will have a significant impact on the dominant pathways and the effect of the strategies applied. Even in the case of ammonia inhibition, which was the topic of this thesis, the use of any given additive in an industrial biogas production process has to take into account many of the factors pointed out throughout this thesis, including the temperature of the process (Papers I, II, III and IV). This parameter is particularly important, as the efficiency of the various strategies studied in this thesis all varied depending on the temperature regimes. Moreover, some of the additives used in this thesis, despite promising, may not be suitable at an industrial scale, as their application may compromise the economic feasibility of the process. Additives like graphene or iron oxides can be an example of such additives (Kalantzis et al. 2023). While graphene's use is not suitable due to its inherently high price, iron oxides are cheap and can be easily produced. Nevertheless, the iron oxide employed in this work was high-grade iron oxide, and not only is high-grade iron oxide not suitable for industrial biogas production given its cost, but also there is no guarantee that cheaper, lower-grade iron oxides will yield the same kind of results seen in this thesis. Nevertheless, the physical characteristics of metal oxides, even if of poor chemical grade, like their conductivity and possibility of making important nutrients readily bioavailable, are a class of materials worth investigating, as many of these materials are also generated as residues of different industries and their possible application in other industrial processes could be a means to valorising them.

One of the most studied additives in this thesis, namely the zeolite clinoptilolite, seems to fit the criteria of a suitable additive to industrial biogas production processes. This additive is a cheap, naturally occurring mineral that can be employed in different particle sizes and, therefore, fit different types of reactor configurations and anaerobic digestion processes

(David L. Bish 2001, Montalvo et al. 2005). Moreover, the fact that it is a naturally occurring mineral and that it possesses ion exchange properties would not only benefit the entire AD and biogas production process by providing additional nutrients and porous structures to the complex microbial communities in these processes, but it would also add value to the digestate resulting from the AD processes employing it, by enriching it with even more important nutrients. Additionally, it could also represent a more efficient, safe and inexpensive means of removing harmful pollutants, like heavy metals, from the digestate, further valuing it (Wang and Peng 2010). Yet, its exploration for the application in biogas production processes implicates its mining, which has environmental implications, making it debatable whether it is an environmentally logical option. Luckily, the prospection of these additives is not exclusively done by mining since there are known technological processes to synthesise them. Moreover, their synthesis in a lab allows their customisation, rendering them even more valuable for specific AD processes and reactor types (Yuna 2016). Yet, it is important to consider the economic feasibility and environmental footprint of the application of both naturally-occurring and laboratory-produced zeolites.

Other additives studied in this thesis that also had positive impacts on SPO, SAO and methane generation were some of the iron and sulfur compounds, namely iron chloride (II) ($FeCl_2$) and, more evidently, iron sulfate ($FeSO_4$) (Paper IV). As previously mentioned, the addition of certain iron compounds, namely iron chloride, is already commonly employed in biogas production processes for purposes other than microbiological ones. However, the results described in this thesis demonstrate that the addition of $FeSO_4$ can benefit biogas production processes operating at thermophilic, high ammonia conditions, even at low levels (Paper IV). In thermophilic biogas production processes operating under high ammonia conditions, the addition of low levels of this compound could be considered as a strategy to overcome high propionate levels, which can persist for long periods in some processes. However, the results from $FeSO_4$ addition in biogas sludge in Paper IV clearly show that this level needs to be carefully fine-tuned with available Fe and S, as well as with the dominant SPO community present in the biogas process, in order to obtain the positive results.

6. Summary Conclusion

In this thesis, the feasibility of applying different strategies for improving syntrophic metabolisms, specifically SPO, was demonstrated for highly enriched, ammonia-tolerant syntrophic cultures. Successful enhancements of SPO, SAO, and hydrogenotrophic methanogenesis were achieved with the addition of graphene, iron oxide nanoparticles, and zeolites, particularly after a second substrate addition. However, during the first addition of propionate to the SPO cultures, a plateau in the propionate degradation profile was observed, likely related to a sudden increase in acetate, H_2, and NH_3 levels. Nevertheless, results from these same cultures indicated that acetate levels below 2 g L^{-1} had a minimal impact on propionate degradation, suggesting that the occurrence of this plateau was probably due to elevated H_2 and NH_3 levels. Moreover, these cultures showed that syntrophic species were found in higher abundance in flocculating communities than in planktonic communities, further underscoring the importance of microbial aggregation for the metabolisms of syntrophic microorganisms. The synergistic effects of temperature, ammonium levels, and zeolite dosages were demonstrated for both mesophilic and thermophilic enrichment cultures. Despite being ammonia-tolerant, increasing ammonia levels still impaired the SPO activity of both mesophilic and thermophilic cultures. Simultaneously, while mesophilic cultures benefited from a slight increase in temperature (within a narrow range) to promote shorter lag phases and enhanced propionate degradation and methane production rates, thermophilic cultures exhibited poorer propionate degradation rates at higher temperatures, possibly due to higher sensitivity to increased ammonia levels. Higher zeolite dosages promoted syntrophic metabolism, particularly by reducing lag phases, lowering acetate accumulation, and increasing methane production rates in the mesophilic enrichments, while enhancing propionate degradation rates in

the thermophilic enrichments. Interestingly, the thermophilic enrichment cultures investigated in this thesis displayed slower propionate and methane production rates compared to their mesophilic counterparts. This trend also applied when iron and sulfur species were supplemented in these enrichment cultures. However, in that experiment, mesophilic enrichment cultures, as well as a mesophilic sludge sourced from an industrial biogas production process, did not benefit from the addition of any iron and/or sulfur compound. In fact, the addition of most iron species inhibited SPO and methane production in the mesophilic enrichment cultures, indicating that iron can be toxic to them. Conversely, the addition of $FeSO_4$ to the thermophilic enrichment cultures significantly enhanced both SPO and methanogenesis. The presence of an SPOB possessing genes related to sulfur reduction metabolism in these cultures indicated a potential benefit from sulfate; however, the addition of Na_2SO_4 did not lead to improved SPO or methanogenesis, suggesting that the positive impact of that compound is instead associated with a combined beneficial effect of both Fe^{2+} and SO_4^{2-}.

This thesis proposes potential strategies for supporting SPOB, SAOB, and their partner methanogens in their metabolisms in high ammonia digesters to enhance industrial biogas processes. The implementation and optimisation of such strategies, supported by predictive models backed by experimental data, can lead to more resilient biogas systems capable of both sustaining episodes of ammonia inhibition and handling high-protein substrates, thereby functioning effectively at elevated ammonia levels.

7. Future Perspectives

Most experiments whose results are presented in this thesis were conducted in batch experiments with ammonia-tolerant, highly enriched syntrophic cultures. The strategies for enhancing syntrophic metabolism include the addition of materials and/or chemical compounds that may not be appropriate for all biogas production processes, depending on several factors, such as the substrate being digested, operating conditions, reactor technology, economic feasibility, and environmental impact. This means that before implementing any of these strategies, smaller-scale experiments must be carried out to replicate, as reliably as possible, the production conditions of the industrial-scale process. To that end, following the results of the present thesis, the addition of the studied materials and/or chemical compounds that have a viable economic and environmental feasibility, like the iron oxide nanoparticles, the zeolites and the $FeSO_4$ solution should be considered for future studies on their impact in SPO and methane production in high-ammonia biogas digesters where syntrophic microorganisms are present in relatively high abundance. As the performance of such digesters depends on the type of substrate being digested and on the microbial community present, and since many of the optimisation strategies employed in this thesis yielded positive results, long-term microbial dynamics studies should be considered to develop a better understanding of how syntrophic microorganisms are impacted by the introduction of these additives in the system. For instance, the addition of $FeSO_4$ has been clearly demonstrated to be capable of improving the propionate-oxidising capacity of a thermophilic culture that was in obvious stress. A long-term microbial community study where an SPO culture is subjected to a sudden episode of stress (such as propionate accumulation) and soon after supplemented with this compound, could potentially unveil the role of specific microorganisms in coping with

these kinds of stress. Moreover, as some of these additives, like iron oxide nanoparticles and zeolites, promote the recruitment of syntrophic species, and aggregation of these species is known to be important for effective IET (Ishii et al. 2005, Weng et al. 2025), long-term studies on the addition of these compounds to digesters operating under high ammonia along with sequential addition of substrate could reveal an even more significant relative abundance of syntrophic microorganisms in these flocculating communities and possibly improved syntrophic metabolism. While DIET has not been unequivocally demonstrated for most SPOB and SAOB, the development of these long-term studies and the employment of metagenomics and metatranscriptomics could give further hints on these syntrophic microorganisms' capacities to perform DIET. Moreover, the power of using predictive models to better understand the relationships between temperature and pH, and their impact on the growth of syntrophic microorganisms, is highly relevant and could, not only help optimise processes where specific syntrophic species are the main acid degraders, but also to characterise the best growing conditions for newly discovered syntrophic species. The potential for improving syntrophic metabolism in high-ammonia biogas digesters paves the way for increasing the OLR in biogas processes treating protein-rich substrates with high methane potential, which can lead to increases in biomethane production and the quality of digestate. Thus, this work could contribute to a greater economic and environmental sustainability of these biogas production processes. While the application of the results described in this thesis to industrial biogas digesters must be approached with cautious expectations, this work establishes a foundation for a better understanding of the metabolism of this important group of microorganisms and highlights potential economically viable strategies for enhancing biogas production in high-ammonia environments.

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Popular science summary

Biogas is produced through the microbial degradation of organic residues in an oxygen-free environment. This process requires the activity of several microorganisms that degrade complex organic materials into smaller compounds, like the acids propionate and acetate, which are key compounds in the degradation chain of organic residues into methane, the energetic chemical present in biogas.

Some types of organic waste have a higher methane potential than others. Protein-rich residues, like slaughterhouse waste and food waste, usually have a high methane potential and are, therefore, desired residues to be degraded. However, during the degradation of these residues, ammonia is produced, which can inhibit several groups of the microorganisms responsible for the transformation of these organic residues into biogas, including the acid-degrading microorganisms that consume propionate and acetate. This can lead to propionate and acetate buildup, which in turn, can result in a cascade of negative effects, including complete process failure.

Yet, when biogas production processes suffer from ammonia inhibition, if given enough time, a new group of acid-degraders tolerant to ammonia and capable of consuming propionate and acetate arise. These acid-degraders, however, have very intricate metabolisms and are dependent on the microorganisms that produce methane to be able to consume propionate and acetate.

This thesis explores different strategies for promoting the activities of these acid-degraders of propionate and acetate in cooperation with methane-producing microorganisms. The studies show that the addition of specific materials like zeolites and iron oxide nanoparticles can help improve the propionate and acetate consumption, and methane production by these microorganisms under high ammonia conditions by bringing them closer

together. The addition of iron and sulfur compounds can also help these microorganisms perform better, particularly for acid-degraders who grow at higher temperatures. For these acid degraders, the addition of iron sulfate was shown to greatly promote the activity of both acid-degraders and methane-producers alike. Computer models can also help improve the growth conditions of these microorganisms by understanding the relationship between important growth parameters, like ammonium and temperature, and zeolite dosages. This is of great importance as it allows us to predict the responses of these microorganisms to changing conditions. In this thesis, acid-degraders consuming propionate and growing at a range of mild temperatures benefited from higher zeolite dosages, particularly at higher temperatures and higher ammonium levels. As for acid-degraders consuming propionate and growing at a range of high temperatures, high dosages of zeolites and lower temperatures were the most beneficial combination, while in the range of ammonium levels where growth happened, ammonium did not exhibit a big impact on their growth.

This thesis highlights different strategies for the promotion of the activity of ammonia-tolerant acid-degraders consuming propionate and acetate in cooperation with methane-producing microorganisms. By promoting their activities, biogas production can become a more resilient and efficient process, especially when degrading protein-rich substrates that give rise to ammonia.

Populärvetenskaplig sammanfattning

Biogas produceras genom mikrobiell nedbrytning av organiska restprodukter i en syrefri miljö. Denna process kräver aktivitet från flera olika mikroorganismer som bryter ner komplexa organiska material till mindre föreningar, såsom syrorna propionat och acetat, vilka är nyckelföreningar i nedbrytningskedjan av organiskt material till metan – den energirika kemiska komponenten i biogas.

Vissa typer av organiskt avfall har en högre metanpotential än andra. Proteinrika restprodukter, såsom slakteriavfall och matavfall, har vanligtvis en hög metanpotential och är därför önskvärda att bryta ner. Under nedbrytningen av dessa restprodukter bildas dock ammoniak, vilket kan hämma flera grupper av mikroorganismer som är ansvariga för omvandlingen av det organiska materialet till biogas, inklusive de syra-nedbrytande mikroorganismer som förbrukar propionat och acetat. Detta kan leda till ansamling av propionat och acetat, vilket i sin tur kan orsaka en kedjereaktion av negativa effekter, inklusive total processkollaps.

När biogasprocesser drabbas av ammoniakhämning kan det, om tillräcklig tid ges, uppstå nya grupper av syra-nedbrytare som är toleranta mot ammoniak och kapabla att förbruka propionat och acetat. Dessa syra-nedbrytare har dock mycket intrikata ämnesomsättningar och är beroende av metanbildande mikroorganismer för att kunna konsumera propionat och acetat.

Denna avhandling undersöker olika strategier för att främja aktiviteten hos dessa syra-nedbrytande mikroorganismer i samarbete med metanproducerande mikroorganismer. Studierna visar att tillsats av specifika material som zeoliter och järnoxid-nanopartiklar kan förbättra konsumtionen av propionat och acetat samt metanproduktionen under höga ammoniaknivåer genom att föra mikroorganismerna närmare varandra.

Tillsats av järn- och svavelföreningar kan också förbättra dessa mikroorganismers prestation, särskilt för syra-nedbrytare som växer vid högre temperaturer. För dessa mikroorganismer visade sig tillsats av järnsulfat kraftigt stimulera aktiviteten hos både syra-nedbrytare och metanbildare.

Datorbaserade modeller kan också användas för att förbättra tillväxtbetingelserna för dessa mikroorganismer genom att förstå sambandet mellan viktiga tillväxtparametrar, såsom ammoniumnivåer, temperatur och zeolittillsatser. Detta är av stor betydelse eftersom det gör det möjligt att förutsäga mikroorganismernas respons på förändrade förhållanden. I denna avhandling gynnades syra-nedbrytare som konsumerar propionat och växer vid milda temperaturer av högre zeolittillsatser, särskilt vid högre temperaturer och ammoniumnivåer. För de som växer vid höga temperaturer visade sig en kombination av höga zeolittillsatser och lägre temperaturer vara mest gynnsam, medan ammoniumnivån inom det intervall där tillväxt skedde inte hade någon större påverkan på tillväxten.

Denna avhandling lyfter fram olika strategier för att främja aktiviteten hos ammoniaktoleranta syra-nedbrytare som konsumerar propionat och acetat i samarbete med metanproducerande mikroorganismer. Genom att främja deras aktivitet kan biogasproduktionen bli en mer motståndskraftig och effektiv process, särskilt vid nedbrytning av proteinrika substrat som ger upphov till ammoniak.

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BIOENERGY AND BIOFUELS

Impact of additives on syntrophic propionate and acetate enrichments under high-ammonia conditions

Eduardo Pinela[1] · Anna Schnürer[1] · Anna Neubeck[2] · Jan Moestedt[1,3,4] · Maria Westerholm[1]

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Abstract

High ammonia concentrations in anaerobic degradation systems cause volatile fatty acid accumulation and reduced methane yield, which often derive from restricted activity of syntrophic acid-oxidising bacteria and hydrogenotrophic methanogens. Inclusion of additives that facilitate the electron transfer or increase cell proximity of syntrophic species by flocculation can be a suitable strategy to counteract these problems, but its actual impact on syntrophic interactions has yet to be determined. In this study, microbial cultivation and molecular and microscopic analysis were performed to evaluate the impact of conductive (graphene, iron oxide) and non-conductive (zeolite) additives on the degradation rate of acetate and propionate to methane by highly enriched ammonia-tolerant syntrophic cultures derived from a biogas process. All additives had a low impact on the lag phase but resulted in a higher rate of acetate (except graphene) and propionate degradation. The syntrophic bacteria '*Candidatus* Syntrophopropionicum ammoniitolerans', *Syntrophaceticus schinkii* and a novel hydrogenotrophic methanogen were found in higher relative abundance and higher gene copy numbers in flocculating communities than in planktonic communities in the cultures, indicating benefits to syntrophs of living in close proximity to their cooperating partner. Microscopy and element analysis showed precipitation of phosphates and biofilm formation in all batches except on the graphene batches, possibly enhancing the rate of acetate and propionate degradation. Overall, the concordance of responses observed in both acetate- and propionate-fed cultures highlight the suitability of the addition of iron oxide or zeolites to enhance acid conversion to methane in high-ammonia biogas processes.

Key points

- *All additives promoted acetate (except graphene) and propionate degradation.*
- *A preference for floc formation by ammonia-tolerant syntrophs was revealed.*
- *Microbes colonised the surfaces of iron oxide and zeolite, but not graphene.*

Keywords Syntrophy · Zeolite · Graphene · Iron oxide · Biogas

Introduction

Nitrogen-rich wastes, such as slaughterhouse waste, animal manure and food waste, are promising substrates for biogas production due to their high methane potential and associated generation of nutrient-rich, high-qualitative fertiliser (Garcia et al. 2019). However, breakdown of nitrogenous compounds in the biogas process gives rise to ammonia (NH_3), a compound that is important for microbial growth but inhibitory at elevated concentrations for some microbial groups (Yenigün and Demirel 2013). In solution, ammonia and its ionised form, ammonium (NH_4^+), exist in equilibrium that is dependent on temperature and pH, with higher temperatures and higher pH shifting the equilibrium towards ammonia, the most toxic of the two compounds

✉ Maria Westerholm
Maria.Westerholm@slu.se

[1] Department of Molecular Sciences, Swedish University of Agricultural Sciences, 750 07 Uppsala, Sweden

[2] Department of Earth Sciences, Uppsala University, 752 36 Uppsala, Sweden

[3] Department of Biogas R & D, Tekniska Verken I Linköping AB (Publ.), Box 1500, 581 15 Linköping, Sweden

[4] Department of Thematic Studies - Environmental Change, Linköping University, 581 83 Linköping, Sweden

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for anaerobic microorganisms (Kayhanian 1999). Elevated ammonia levels can affect many microbial groups responsible for the early stages of anaerobic digestion, such as hydrolysis and acidogenesis. In later stages, volatile fatty acids (VFA) such as propionate and acetate are converted into smaller compounds such as acetate (in the case of propionate degradation), formate, H_2 and CO_2. The negative impact of ammonia on process performance and biogas yield derives mainly from its impact on methanogens, which are responsible for the last step in biogas production, with aceticlastic methanogens, which utilise acetate to produce CO_2 and methane, being particularly sensitive (Yenigün and Demirel 2013). Ammonia inhibition can thus lead to accumulation of VFAs, decreased methane yield and reduced overall process efficiency (Rajagopal et al. 2013; Jiang et al. 2019). Under high-ammonia conditions, aceticlastic methanogens are outcompeted by ammonia-tolerant microorganisms, such as syntrophic acetate-oxidising bacteria (SAOB), which convert acetate to CO_2 and formate/H_2 (Westerholm et al. 2011a, b; Wang et al. 2015). However, these microorganisms cannot perform acetate oxidation if formate/H_2 levels are too high and therefore require a close association with hydrogenotrophic methanogens, which consume and maintain formate/H_2 at low levels, preventing acetate accumulation (Westerholm et al. 2015; Bonk et al. 2018; Singh et al. 2021). Another VFA that easily accumulates under high ammonia concentration is propionate, which is converted by syntrophic propionate-oxidising bacteria (SPOB) both under high- and low-ammonia conditions (albeit by different species) into CO_2, formate/H_2 and acetate. Similarly to acetate oxidation by SAOB, propionate oxidation by SPOB requires a close association with hydrogenotrophic methanogens to avoid thermodynamic constraints (Dolfing 1992; Si et al. 2005; Westerholm et al. 2022). The reason for propionate accumulation under high ammonia concentrations remains unclear, but it may be caused by direct ammonia inhibition of certain SPOB (Bonk et al. 2018; Zhang et al. 2018). However, even when an ammonia-tolerant SPOB is present, propionate degradation can be hampered due to ammonia-related stress on SPOB or indirectly through reduced methanogenic activity (Liu et al. 1999; Westerholm et al. 2022), resulting in build-up of acetate and propionate and loss of methane potential in high-ammonia processes.

It has been suggested that the VFA degradation rate in anaerobic processes can be improved by facilitating and increasing exchange of the electron-carrying intermediary compounds formate (Thiele and Zeikus 1988) and H_2 (Dolfing 2013), by reducing cell-to-cell distances between syntrophic microorganisms. Another mechanism for electron allocation between different microbial species is the direct interspecies electron transfer (DIET) through e-pili (Reguera et al. 2005) or outer-membrane transport proteins (Nevin and Lovley 2000). Even though DIET has been proposed to provide a slight thermodynamic advantage compared to using intermediary electron-carrying compounds (Storck et al. 2016), it has been suggested that DIET and interspecies formate/H_2 transfer can occur simultaneously (Jing et al. 2017). This would theoretically benefit syntrophic activity as it offers different strategies to transfer electrons (Viggi et al. 2014). Several SPOB and SAOB have been shown to use both formate and H_2 for electron transfer (Hidalgo-Ahumada et al. 2018; Singh et al. 2023; Weng et al. 2024) but only *Pelotomaculum thermopropionicum* has been suggested to perform DIET, by means of conductive nanowires (e-pili) (Gorby et al. 2006), and no SAOB has yet been proven to have this capability. Similar to the use of intermediary compounds, also DIET would theoretically be promoted by cell proximity and syntrophic communities have been found aggregated in different ways (Thiele et al. 1988; Ishii et al. 2005; Weng et al. 2024), which could be a strategy of the involved microorganisms to reduce distances. Reduction in cell distance has also been proposed to be facilitated by presence of additives like zeolites that function as a platform to which the microorganisms can adhere and establish direct and indirect connections (Montalvo et al. 2012). Furthermore, some zeolites have the additional benefit of being capable of exchanging ions with toxic compounds (such as ammonia) present in the media (Wang and Peng 2010). It has also been suggested that the electrical conductivity in some additives (like graphene and iron oxide) can serve as an electron-shuttling mechanism and thereby facilitate DIET between cells (Rotaru et al. 2014; Zhuang et al. 2018). Even though the inclusion of additives has been shown to enhance the overall anaerobic digestion process (Wang et al. 2018a, b; Li et al. 2019), few studies have explicitly demonstrated that this impact derives from promotion of syntrophic acid-degrading microorganisms. This is because, in biogas processes, determining acid formation and degradation rates is complicated by the constant formation of VFAs resulting from breakdown of compounds higher up in the degradation chain. Furthermore, despite having central roles in biogas processes, syntrophs typically constitute only a few percent of the total microbial community, making them difficult to study (Westerholm et al. 2016).

The aim of the present work was to specifically examine how the presence of additives impact on ammonia-tolerant syntrophic microorganisms, with regard to their acid-degrading capacity and floc formation. This was conducted using highly enriched microbial communities of acid-oxidising bacteria (SAOB and SPOB) and hydrogenotrophic methanogens, originating from a high-ammonia biogas digester. Cultivations were conducted in batch assays under high (0.3 M) ammonia and mesophilic (37° C) conditions, with either the conductive additives graphene powder or iron oxide (II, III) nanoparticles, or the non-conductive zeolite clinoptilolite. Acetate and propionate degradation and methane formation

were analysed and the impact of the additives on microbial community composition was determined for flocculating and planktonic microbial communities. Microbial samples were analysed after the batch cultivations using scanning electron microscopy (SEM) and element analysis, to further reveal the disposition of cells attached to the additives and to assess the formation of precipitates.

Material and methods

Source of syntrophic communities and anaerobic batch set-up

Enriched syntrophic acetate-oxidising (SAO) and syntrophic propionate-oxidising (SPO) communities used as inoculum cultures were obtained from laboratory-scale, continuously stirred tank reactors operating under anaerobic and mesophilic conditions (37 °C) as described in Singh et al. (2021). In short, the reactors were inoculated with sludge from a high-ammonia biogas process (5.4 g NH_4^+-N L^{-1}, 0.6–0.9 g NH_3 L^{-1}) and continuously fed with bicarbonate-buffered basal medium (BM) containing 0.2 g L^{-1} yeast extract, vitamins and trace elements, two reductants to remove traces of oxygen, cysteine-HCl (0.5 g L^{-1}) and Na_2S (0.24 g L^{-1}) (Westerholm et al. 2010), 0.1 M sodium acetate (SAO enrichment cultures) or sodium propionate (SPO enrichment cultures) and 0.3 M ammonium chloride (16 g NH_4Cl L^{-1}). At the end of the process, the microbial cultures were transferred from the reactors to anaerobic batch flasks while flushing with N_2.

A cultivation experiment was conducted in 1-L anaerobic batch flasks containing 0.45 L of the aforementioned BM in anoxic conditions but with 0.3 M ammonium chloride (pH 7.3, 4.7 g NH_4^+-N L^{-1}, 0.1–0.2 g NH_3 L^{-1}) and 50 mM sodium acetate (4.1 g L^{-1}) or 50 mM sodium propionate (4.8 g L^{-1}). In the iron oxide nanoparticle batches, 25 mg L^{-1} of iron oxide ($Fe(II,III)_3O_4$) nanoparticles (Sigma-Aldrich) was added; in the graphene batches, 25 mg L^{-1} of graphene nanoparticles (Sigma-Aldrich) was added; and in the zeolite batches, 5 g L^{-1} of zeolites (clinoptilolite; Zeo-Concept Ece AB) was added, under continuous N_2 flux. After the inclusion of these additives, all flasks were closed with a rubber stop and an aluminum ring, and the gas phase was swapped to a gas mixture of CO_2 (19.9%) and N_2 (80.1%) at an overpressure of 0.2 atm. After autoclavation at 121 °C, for 20 min, the flasks were allowed to cool down to room temperature and 25 mL of each of the two abovementioned reductants cysteine-HCl (0.5 g L^{-1}) and Na_2S (0.24 g L^{-1}) was syringe-filtered to all batches. All sets of batches were established in triplicate and triplicate control batches (without additives) were included. The batches were inoculated with 5% (v/v) of the enrichment cultures described above. The inoculum cultures were thoroughly shaken prior to inoculation to ensure a heterogeneous content and homogeneous number of cells. The set of batches with acetate as substrate was inoculated with the SAO enrichment cultures while the set of batches with propionate as substrate was inoculated with the SPO enrichment cultures. All batches were then incubated in a dark room at 37 °C without shaking. After a first period of complete acid degradation (referred to as A1 for acetate degradation and P1 for propionate degradation), all batches were starved for at least 14 days to ensure absence of acid-degrading activity, and once again fed with the respective acid in a sterile stock solution to reach 50 mM in the culture media. The batches were then again incubated at 37 °C and a second complete acid degradation (referred to as A2 for acetate degradation and P2 for propionate degradation) was performed.

In order to investigate the impact of acetate on propionate degradation, a complementary batch trial was performed without inclusion of additives. Batch flasks with medium containing 0.3 M NH_4Cl and 95 or 47 mM propionate were prepared as described above. At both propionate levels, 0 (control), 14, 29 or 61 mM acetate were added before inoculation with 5% (v/v) of the SPO enrichment culture. All batch sets were prepared in triplicate (see Table S1 in Supplementary Information (SI)) and incubated at 37 °C without shaking.

Analytical methods

Propionate and acetate levels were analysed using high-performance liquid chromatography (HPLC) (threshold for acid detection was 0.2 g L^{-1}) and methane and CO_2 contents were assessed through gas chromatography (GC) as described by Westerholm et al. (2010). During A1 and P1, H_2 partial pressure measurements (pH_2) were made using PP1 (Peak Performer 1, reduced gas analyser) by direct injection of 1 mL of gas sample withdrawn from the headspace of the batch flasks. Standard curves using different pH_2 were prepared prior to injection of samples. During A1 and P1, HPLC and GC analyses, and H_2 and pressure readings were performed weekly and pH measurements every 2 weeks. During A2 and P2 and in the trials investigating the impact of acetate on propionate degradation, pressure readings and HPLC and GC analyses were performed weekly.

Acid degradation and carbon balance calculations and statistical significance

Acid degradation rates were calculated by including the linear degradation phase (highlighted in Fig. 1a and b and Fig. 2a and b) using the equation:

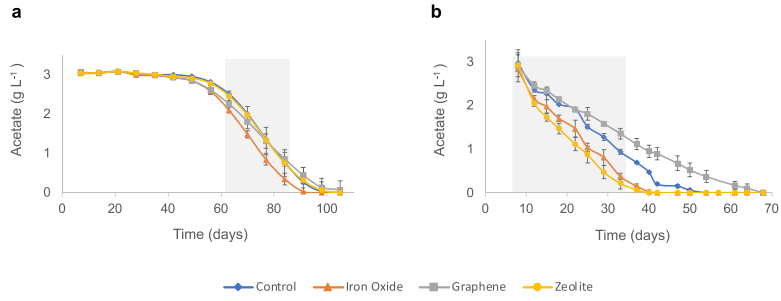

Fig. 1 Degradation curves for the batches without (control) and with additives (iron oxide, graphene, zeolite) during **a** the first (A1) and **b** the second (A2) round of acetate degradation. Shaded areas indicate location of data points used for degradation rate calculations

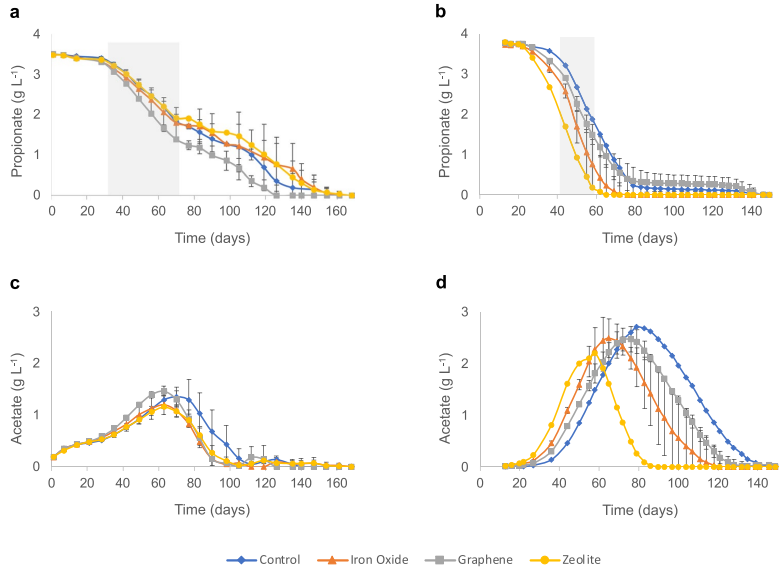

Fig. 2 Propionate and acetate degradation curves during **a**, **c** the first (P1) and **b**, **d** the second (P2) round of propionate degradation. Shaded areas indicate location of data points used for calculation of degradation rate. Mean of triplicates ± standard deviation for all except the control and zeolite batches in **b** and **d**, which are mean of duplicates

$$\text{Rate of acid degradation } \left(\text{g L}^{-1}\text{day}^{-1}\right) = \frac{\left(\text{Ci } \left(\text{g L}^{-1}\right) - \text{Cx } \left(\text{g L}^{-1}\right)\right)}{\Delta \text{ days}} \quad (1)$$

where Ci is concentration at the start of the degradation phase and Cx is concentration at a defined time point of the linear degradation phase (see Table S2 in SI for details). Carbon balance calculations were made based on the oxidation reactions of acetate or propionate to methane, which gave a theoretical conversion coefficient of 1 (Ferry 1992) and 1.75 (Singh et al. 2023), respectively (see Table S3 in SI for details). Statistical significance was assessed by single-factor ANOVA, with significance threshold set at $p < 0.05$.

Sampling, DNA extraction and 16S rRNA gene sequencing

To avoid disturbing the syntrophic communities and acid degradation during the experiments, samples from the experimental batches were only taken at the end of A2 and P2. Microbes residing in flocculating communities were sampled by inverting the bottles to allow flocculating communities to sink to the inner side of the rubber stopper of the anaerobic flask, from which samples were withdrawn. Samples of planktonic cells were taken from the upper part of the liquid medium by gently tilting the flask halfway,

leaving the flocs on the bottom of the flask. Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) and 16S rRNA gene sequencing was performed as described by Müller et al. (2016) using the primers 515F (5′-GTGBCAGCMGCCGCGGTAA-3′) and 805R (5′-GGACTACHVGGGTWTCTAAT-3′) (Hugerth et al. 2014). Paired-end sequencing was performed on an Illumina MiSeq instrument (Eurofins GATC Biotech GmbH, Germany) at SciLifeLab Stockholm, Sweden. Cutadapt v 4.2 was used to trim Illumina adapters and primer sequences (Martin 2017). Amplicon sequence variants were generated and their taxonomic assignments and abundance tables were determined using the package dada2 (v. 1.26.0) (Callahan et al. 2016) in R (v. 4.2.2). Taxonomic classification of the 16S rRNA gene sequence variants was performed using the Silva taxonomic training dataset v138.1 formatted for DADA2 (McLaren 2020). A phyloseq object was created using abundance and taxonomy tables for visualisation of community structure with the package phyloseq (version 1.42.0) (McMurdie and Holmes 2013) in RStudio version 2022.12.0 + 353 (Posit Software, PBC, 2022).

Quantitative polymerase chain reaction (qPCR)

Separate qPCR analyses targeting the 16S rRNA genes of ammonia-tolerant SPOB, SAOB and methanogens were set up in order to screen for their gene abundance in the flocculating and planktonic communities. To that end, the primers MMBf (5′-ATCGRTACGGGTTGTGGG-3′) and MMBr (5′-CACCTAACGCRCATHGTTTAC-3′) (Yu et al. 2005) targeting the order *Methanomicrobiales* and THACf (5′-ATCAACCCCATCTGTGCC-3′) and THACr (5′-CAGAATTCGCAGGATGTC-3′) targeting the SAOB *Syntrophaceticus* schinkii (Westerholm et al. 2011a, b) were used. For targeting the SPOB '*Candidatus* Syntrophopropionicum ammoniitolerans', novel primers were designed, since existing species-specific primers (Singh et al. 2021) did not encompass all 16S rRNA gene copies of this candidate SPOB, according to the NCBI annotation. Hence, primers SPAf (5′-CCACAGCCTGCCTTTGAAAC-3′) and SPAr (5′-CGTCAGAAACAGGCCAGAGA-3′) were designed as specified previously (Singh et al. 2021). Construction of DNA standard curves was performed as described in Westerholm et al. (2011a, b). The qPCRs were performed in a 20-µL reaction mixture that consisted of 3 µL DNA sample, 10 µL iQ™ SYBR® Green Supermix (Bio-Rad), 1 µL of each primer (10 pmol μL^{-1}) and 5 µL UltraPure™ distilled water (Invitrogen). The qPCR protocol for quantification was as follows: 7 min at 95 °C, 40 cycles of 95 °C for 40 s, annealing at 66 °C, 61 °C or 59.3 °C (for the order *Methanomicrobiales*, species *S. schinkii* and species '*Ca* S. ammoniitolerans', respectively) for 1 min and 72 °C for 40 s, and melting curve analysis at 95 °C for 15 s, followed by 1 min at 55 °C and finally at 95 °C for 1 s. All reactions were carried out in QuantStudio™ 5 (ThermoFisher).

Scanning electron microscopy

Samples for SEM were taken from one batch flask of each set of treatment triplicates at the end of A2 and P2. The flasks were opened in an anaerobic box and their rubber stoppers replaced with an adapted rubber stopper with a glass vial inserted in it. To collect undisturbed cultures from the media, the batch flasks were gently tilted to allow the microorganisms to enter the glass vial. The glass vial was then removed from the rubber stopper and closed. Prior to imaging, the samples were filtered (glass fibre filter) with vacuum suction to remove excess liquids. Cell fixation was performed by soaking the filters in a mixture of 4 mL glutaraldehyde, 4 mL phosphate buffer (1 M) and 32 mL deionised water for 4 h, following by soaking in 0.1 M phosphate solution for 10 min. This step was repeated three times, followed by drying with ethanol (50%, 70%, 80%, 90%, 95% and 100% v/v) for 10 min each. The 100% v/v ethanol wash was repeated three times. After the final ethanol drying, the filters were placed in hexamethyldisilazane for 5 min and then left to air-dry in a desiccator overnight. All samples were coated with palladium/gold prior to SEM analysis, which was performed in Geocentrum, Uppsala University, using a Zeiss Supra 35 VP field emission SEM (Carl Zeiss SMT, Oberkochen, Germany) equipped with a variable pressure scanning electron low-vacuum detector and a Robinson backscatter detector. Microphotographs were taken using a Leica MZ75 optical light microscope mounted with a Nikon digital sight DS U1 and NIS-Elements F2.20 software. Images were taken with a beam setting of 4 kV and an aperture of 30.0 µm at an optimal working distance of 8.5 mm. Element analyses were performed using an energy-dispersive X-ray analysis (EDAX) Apex 4 device (Ametekh, Mahwah, USA) coupled with an energy-dispersive X-ray spectroscopy (EDS) detector for X-ray microanalysis.

Results

Acetate degradation in the presence of additives

During period A1, the lag phase lasted 49 days for the iron oxide batches and 56 days for all others, after which the added acetate was degraded at relatively similar rates in all sets of batches except those supplemented with graphene, which had a slower degradation rate, though not significant ($p > 0.5$) (Fig. 1a, Table 1). The acetate level decreased to below the detection limit in the iron oxide batches at day

Table 1 Lag phase, rate of degradation and day on which acid level was below the detection limit (< 0.2 g L^{-1}) in the first and second rounds of acid degradation. Rate of acetate degradation was calculated using values from between days 63 and 84 for A1 and between days 8 and 33 for A2. Rate of propionate degradation was calculated using values from between days 35 and 70 for P1 and between days 44 and 58 for P2. Mean of triplicates and standard deviation are presented, or otherwise mean of duplicates

	First round (A1, P1)			Second round (A2, P2)		
	Lag phase (days)	Rate of degradation (g L^{-1} day^{-1})	Acid below detection (day)	Lag phase (days)	Rate of degradation (g L^{-1} day^{-1})	Acid below detection (day)
Acetate						
Control	56	0.083 ± 0.000	98	8	0.081 ± 0.004	47
Iron oxide (II, III)	49	0.083 ± 0.001	91	8	0.098 ± 0.003[b,c]	40
Graphene	49	0.067 ± 0.010[b]	98	8	0.062 ± 0.009[b]	64
Zeolite	56	0.081 ± 0.004	98	8	0.11 ± 0.012[b,c]	37
Propionate						
Control[a]	28	0.040 ± 0.001	135	36	0.096 0.095	83
Iron oxide (II, III)	28	0.038 ± 0.007	154	27	0.130 ± 0.011[c]	69
Graphene	28	0.048 ± 0.002[b]	119	27	0.100 ± 0.006[c]	135
Zeolite[a]	28	0.037 ± 0.001[b]	147	22	0.095 0.122	58

[a]Standard deviation not calculated in the second round due to loss of one of the triplicate batches

[b]Significant compared to the control of the corresponding round

[c]Significant compared to the correspondent batch during the first round of acid degradation

91 and in the other batches at day 98 (Table 1). Measured H_2 partial pressure varied between 2 and 14 Pa and the pH increased from 7.4 to 8.2 over the course of the experiment (Fig. S1, Fig. S2). The higher pH over time resulted in an increased proportion of free ammonia in the batches (see Table S4 for details), from 0.13 to 0.7 g NH_3 L^{-1} at the end of A1 (Fig. S3a).

After feeding with an additional 50 mM of acetate (A2), the lag phase of all batches was 8 days (Table 1). Notably, the iron oxide ($p < 0.01$) and zeolite ($p < 0.04$) batches had significantly faster degradation rates in A2 than in A1, a trend not observed in the graphene and control batches. Hence, in A2, the acetate levels fell below the detection limit after 37 and 40 days for the iron oxide and zeolite batches, respectively, and after 47 and 64 days for the control and graphene batches, respectively (Fig. 1b, Table 1). The SAO community showed a significantly faster acetate degradation rate in the presence of iron oxide ($p < 0.02$) and zeolites ($p < 0.04$) than in the control, while the presence of graphene significantly hampered acetate degradation rate ($p = 0.049$).

Propionate degradation in the presence of additives

During period P1, all SPO-enriched batches had a lag phase of 28 days (Table 1). All batches displayed similar propionate degradation profiles, consisting of two phases of linear degradation (between days ~ 30–70 and 100–120/150). At the point at which acetate level began to decrease (day 70 for the controls, day 63 for all other batches), a phase of slower propionate degradation was observed (days ~ 70–100), i.e. when propionate and acetate level was around 1.5–2 g L^{-1} and 1.2–1.5 g L^{-1}, respectively (Fig. 2a and c). During this intermediary lag phase, H_2 partial pressure increased from 3.5–5 to 5.5–7.5 Pa (Fig. S4) and pH increased from 7.6 to 7.7–7.9 (Fig. S5). The latter resulted in temporary increased ammonia level (see Table S4 for details), from 0.2 to 0.3–0.4 g L^{-1} (Fig. S3b). A phase of faster propionate degradation coincided with lowering of the ammonia concentration (from 0.4 to 0.2 g L^{-1}) and H_2 level (from 6–7 to 1–3 Pa), where they remained stable until the end of the experiment. The molar conversion ratio of propionate into acetate did not reach 1:1 at the peak acetate level (except for the graphene batches), indicating that the SAO community was active and degraded acetate concurrently with its formation (Fig. S6). However, the accumulation of acetate up until days 63–70 indicated that acetate degradation by the SAOB was not sufficiently fast to match the rate of propionate degradation by the SPOB. Following the peak in acetate levels, both acetate and propionate were consumed until they dropped below the detection limit.

Regarding the impact of additives on propionate degradation, inclusion of graphene had a significant ($p = 0.005$) positive impact on the degradation rate during P1, whereas the presence of iron oxide did not have a significant effect ($p > 0.06$) and the zeolite significantly ($p < 0.05$) lowered the degradation rate (Table 1, Fig. 2a). Accordingly, in the presence of graphene, propionate was degraded to below the detection limit after 119 days, whereas the batches with other amendments required 135–154 days to deplete all propionate. The acetate level in all sets of batches was below the detection limit before day 105 (Table 1, Fig. 2a and c). The H_2 partial pressure during P1 varied between 1

and 7 Pa (Fig. S4), which was somewhat lower than in A1. The pH varied between 7.4 and 7.9 in all batches during P1, which was a lower pH increase than in the acetate-fed batches (Fig. S5). This resulted in the concentration of ammonia increasing from 0.12 to 0.32 g NH_3 L^{-1} at the end of P1 (Fig. S3b).

In contrast to the acetate experiment, the lag phase in P2 was similar to that in P1, varying between 22 and 36 days (Table 1). Interestingly, a different degradation curve to that in P1 was observed after the second feeding of propionate. During P2, all batches exhibited a single faster degradation phase (Fig. 2b) and a higher rate of propionate consumption than in P1, which was highest for the iron oxide batches, followed by the zeolite batches, and for the graphene and control batches, which had similar degradation rates (Table 1). These differences were statistically significant ($p < 0.001$) for both iron oxide and graphene batches, while for the control and zeolite batches the significance of these differences was not possible to determine. Throughout P2, acetate concentration built up to a peak at around 2.3–2.8 g L^{-1}, after which it was steadily consumed to below the detection limit (Fig. 2d). The peak approximately coincided with the time point at which the propionate level was below the detection limit for the iron oxide and zeolite batches, while for the control and graphene batches, the point at which acetate level began to decrease (day 79 for the controls, day 76 for the graphene batches), a phase of slower propionate degradation was observed (Fig. 2b and d). In P2, the acid levels in the zeolite batches were below detection after 80 days, whereas in the other batches acids were fully consumed at between 111 and 135 days (Table 1, Fig. 2b and d).

Cultivation experiments conducted to investigate the impact of acetate on propionate degradation rate and lag phase in the absence of additives showed that presence of acetate up to 2 g L^{-1} had a low impact on propionate degradation rate of the SPO enrichment culture during degradation of 3.5 g L^{-1} propionate (Fig. S7). However, in the presence of 4 g L^{-1} acetate, propionate was degraded at a reduced rate. In the SPO culture initiated at 7.5 g L^{-1} propionate, acetate concentrations of 1 g L^{-1} and above decreased the propionate degradation rate (Fig. S7).

Methane formation and carbon balance calculations

In A1, 22–24 mmol of methane was formed, corresponding to a yield of 88–96% based on the carbon balance calculations (Fig. S8a). In A2, all batches produced 18–20 mmol of methane. Hence, about 72–80% of the acetate present was converted to methane in A2, which was significantly ($p < 0.01$) lower than in A1 (Fig. S8b). During P1, the carbon balance calculations demonstrated equimolar conversion of 25 mmol propionate into methane (39–40 mmol, i.e. 98–100% yield) (Fig. S9a). In P2, 30–31 mmol of methane was produced, which represented a significantly ($p < 0.01$) lower conversion yield for all batches (75–78%) in comparison with P1 (Fig. S9b).

Microbial community structure

Analysis of microbial community composition by Illumina sequencing targeting the 16S rRNA gene revealed differences in community composition, particularly between the acetate and propionate experiments, but also between the flocculating and planktonic microbial communities (Fig. 3). In the acetate-fed batches, the most abundant genus was *Alkaliphilus*, with relative abundance varying from 53 to 74% in all batches and with no discernible differences between flocculating and planktonic communities. Another species present in all batches was the SAOB *S. schinkii* (99% sequence similarity based on 16S rRNA gene sequence). Interestingly, this SAOB was present in higher relative abundance in the flocculating communities (3–17%) than in the

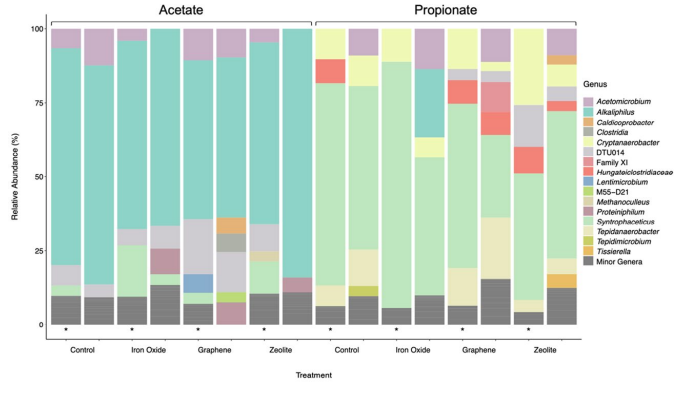

Fig. 3 Microbial community structure at genus level in the flocculating (bars marked with an asterisk) and planktonic communities in the acetate- and propionate-fed batches. All samples were taken at the end of the second round of acid degradation (A2, P2). The threshold for representation in the image was 3%. The sequence assigned as *Cryptanaerobacter* was 99% similar to the 16S rRNA gene sequence of '*Ca* S. ammoniitolerans' (Singh et al. 2021)

planktonic communities (< 3–4%), although the difference was not statistically significant ($p = 0.073$) (Fig. 3). Members of the unclassified DTU014 and the genus *Acetomicrobium* were also detected (< 3–25%) in all communities except the planktonic communities in the zeolite and iron oxide batches.

In the propionate-fed batches, the predominant species in all samples was *S. schinkii* (Fig. 3). Again, this species was present in higher relative abundance in the flocculating communities (43–83%) than in the planktonic communities (28–55%), but the difference was not significant ($p = 0.066$). Another genus present in all propionate-fed batches was *Cryptanaerobacter*. Sequence comparison revealed this to be '*Candidatus* Syntrophopropionicum ammoniitolerans', previously identified as a candidate SPOB in the reactors from which the inoculum originated (99% similarity based on sequence alignment of the 16S rRNA gene retrieved from MAG62 in Singh et al. (2021), accessible at GenBank JABMJD000000000, hereafter referred to as '*Ca* S. ammoniitolerans') (Singh et al. 2021). This candidate SPOB was present at higher relative abundance in the samples taken from flocs (10–26%) than in those taken from planktonic microorganisms (3–10%), although this difference was not statistically significant ($p = 0.058$). Furthermore, a species previously known as an ammonia-tolerant SAOB, *Tepidanaerobacter acetatoxydans* (Westerholm et al. 2011a, b) (96% similarity based on nucleotide sequence blast of the 16S rRNA gene), was present above the detection threshold in all propionate-fed batches except the iron oxide batches. The relative abundance of this species was 4–13% in the flocculating communities, whereas it represented 5–21% of the planktonic communities (Fig. 3). Also present at higher relative abundance in the flocculating communities (< 3–16) than in the planktonic communities (< 3–10%) (except in the graphene batches) was the family *Hungateiclostridiaceae*. In the zeolite batches, the unclassified DTU014 was detected at higher relative abundance in the flocculating community (3–30%) than in the planktonic community (4–6%). Another notable finding was higher relative abundance of *Acetomicrobium* in the planktonic community (3–26%) than in the flocculating community (< 3–5%) in the propionate-fed batches (Fig. 3). Relative abundance of a methanogenic partner only exceeded the threshold in the zeolite-amended, acetate-fed flocculating community. From this sample, genes affiliated to the methanogenic genus *Methanoculleus* were obtained.

Quantitative abundance of SPOB, SAOB and hydrogenotrophic methanogens in flocculating and planktonic communities

In the SPO enrichment cultures, quantification of methanogens using qPCR revealed significantly higher levels of hydrogenotrophic methanogens belonging to the order *Methanomicrobiales* in the flocculating communities than in the planktonic communities in all batches ($p < 0.01$). In the SAO enrichment cultures, only the control had significantly higher levels of methanogens in the flocculating communities ($p < 0.02$) than in the planktonic communities, while the batches treated with iron oxide had higher levels in the planktonic communities than in the flocculating communities (difference not significant; $p > 0.8$) (Fig. 4d and e). The graphene- and zeolite-amended batches had, respectively, lower and higher methanogen levels in their flocculating communities than in planktonic communities, but the significance of these differences could not be confirmed. Similarly, the SAOB *S. schinkii* was present in higher levels in flocculating communities than in planktonic communities in the acetate-fed, graphene- and zeolite-amended batches, but this difference could not be statistically verified. However, the zeolite batches of the acetate-fed cultures demonstrated significantly ($p < 0.05$) higher levels of this SAOB in their flocculating communities than the control batches. This SAOB was also present in significantly ($p < 0.01$) higher levels in flocculating communities than in planktonic communities of the control, but not of the iron oxide batches ($p > 0.5$), in the acetate experiment (Fig. 4c). In the propionate-fed batches, this SAOB was present in significantly higher levels in the flocculating communities than in the planktonic communities of all batches ($p < 0.03$), with the flocculating communities of both the graphene- and zeolite-amended batches displaying significantly ($p < 0.03$) higher SAOB levels than those of the control (Fig. 4b). The SPOB '*Ca* S. ammoniitolerans' was also present in significantly higher levels in flocculating communities than in planktonic communities of the propionate-fed batches ($p < 0.02$) (Fig. 4a). For this organism, only the flocculating communities of the iron oxide batches had significantly higher values than the control ($p < 0.05$).

Scanning electron microscopy

The SEM images of samples from the iron oxide-supplemented batches showed densely packed microbial communities attached to the iron oxide grains (Fig. 5a–c). Element analyses using EDAX and EDS demonstrated presence of pyrites (FeS_2, cubic) as well as phosphate precipitates (vivianite) in both the acetate- and propionate-degrading batches supplemented with iron oxide nanoparticles (Fig. 5d).

In contrast to the extensive surface colonisation visible in the samples from iron oxide-supplemented batches, the images of the acetate- and propionate-fed cultures with added graphene showed few scattered cells attached on the grain surface and attachment of cells in between the graphene grains (Fig. 6). No extensive phosphate precipitation or other secondary minerals were detected in the samples from the cultures with graphene addition.

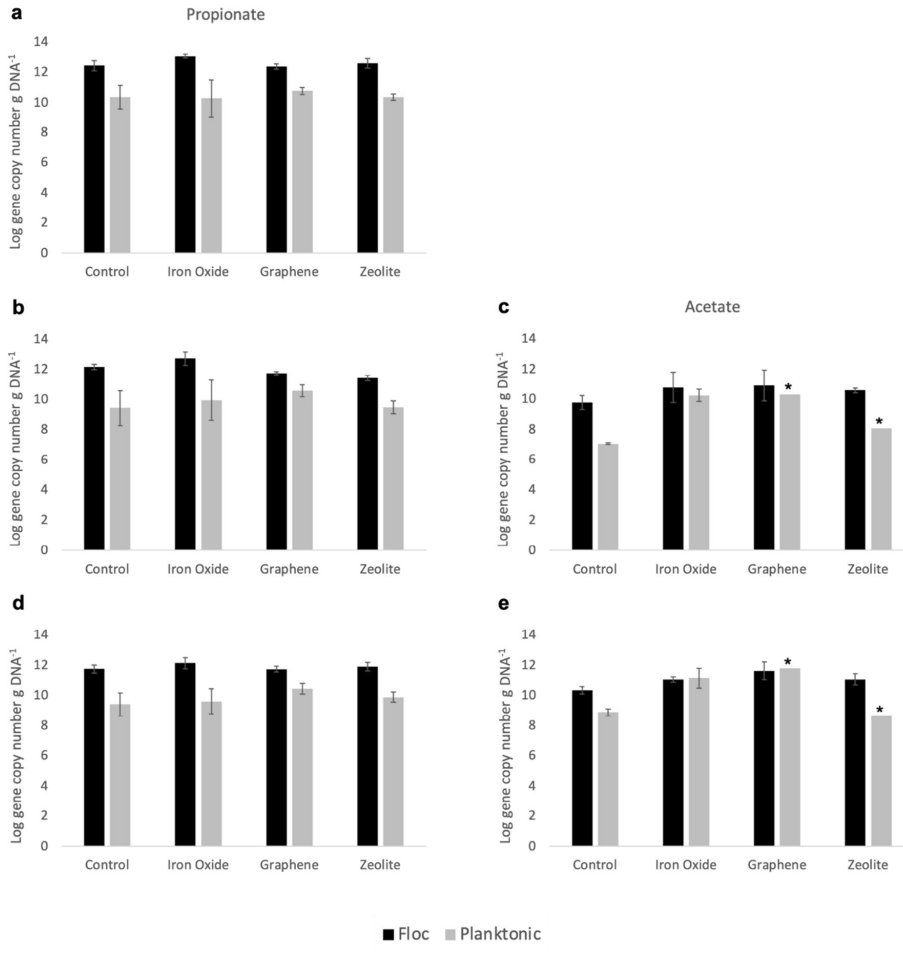

Fig. 4 Gene copy number of **a** the syntrophic propionate-oxidising bacteria '*Ca* S. ammoniitolerans', **b**, **c** the syntrophic acetate-oxidising bacteria *S. schinkii* and **d**, **e** the partner hydrogenotrophic methanogen in the flocculating and planktonic communities of the propionate (left panels) and acetate (right panels) degradation experiments. All values are mean of triplicates except those marked with and asterisk, which are mean of duplicates

All SEM images of the cultures supplemented with zeolite showed extensive precipitation of phosphates (Ph) of different crystal morphologies (Fig. 7a–h). In the sample from propionate-degrading cultures, prism-like (Fig. 7a–e), rosette-like rounded (Fig. 7a, c, d and g) and irregular (Fig. 7e, h) calcium and magnesium phosphates were visible. The microorganisms colonised the surface and formed biofilm on all types of phosphate precipitates (Fig. 7h). The EDAX and EDS analyses revealed no precipitates other than phosphates in the zeolite cultures.

Discussion

Propionate and acetate degradation dynamics and carbon balance were altered after second addition of acids

Irrespective of the presence of additives, all propionate-degrading batches showed faster acid consumption during the second substrate degradation (P2) compared with the first (P1), while the acetate batches had a shorter lag phase

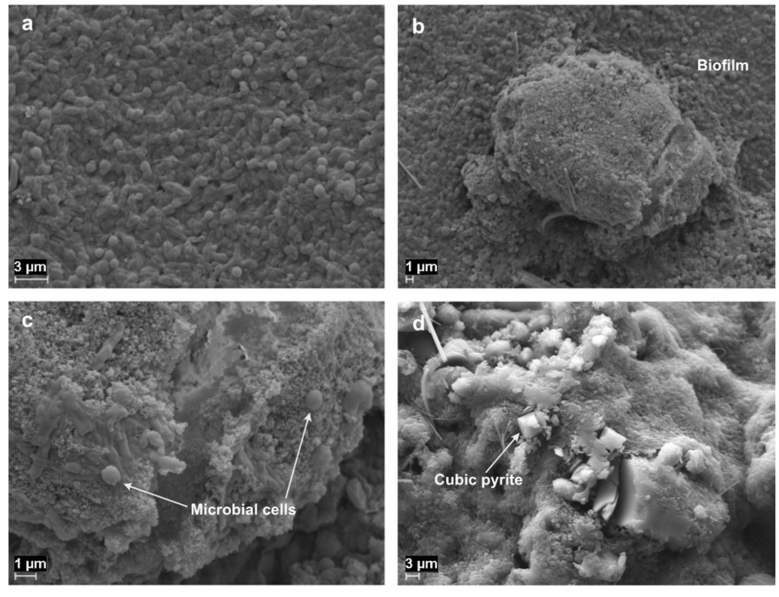

Fig. 5 SEM images of the cultures supplemented with iron oxide. Images **a**–**c** were obtained from propionate-degrading cultures, while image **d** was obtained from acetate-degrading cultures

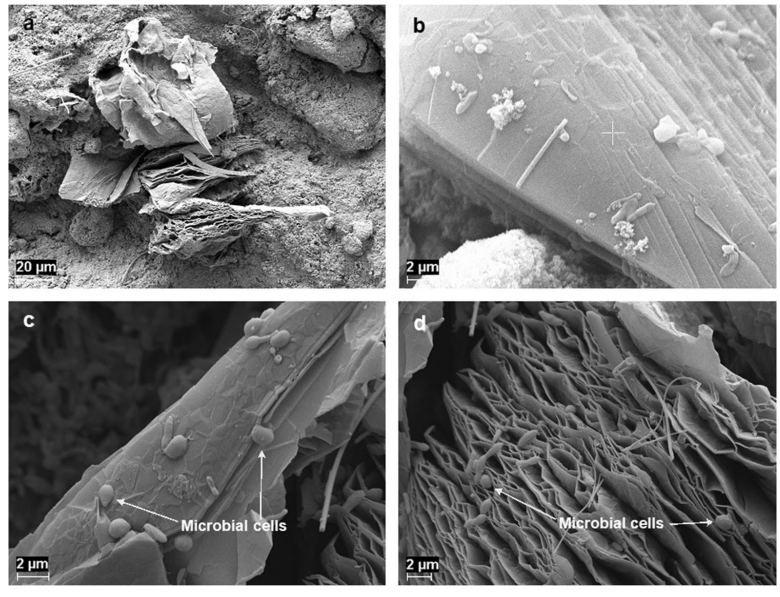

Fig. 6 SEM images of the cultures supplemented with graphene. Images **a** and **b** were obtained from propionate-degrading cultures, while images **c** and **d** were obtained from acetate-degrading cultures

in the second substrate degradation (A2) compared with the first (A1) (Figs. 1 and 2, Table 1). This is likely explained by the visibly higher abundance of microorganisms and flocs (Fig. S10c and b) and by metabolically important enzymes having already been formed at this point, but not at the beginning of A1/P1 (Fig. S10a and b) (Thiele et al. 1988; Brock et al. 2003). Another conceivable explanation is that the higher pH levels (7.9/8.2) observed at the beginning of the second round of degradation may have been more conducive to growth of syntrophic communities than the lower

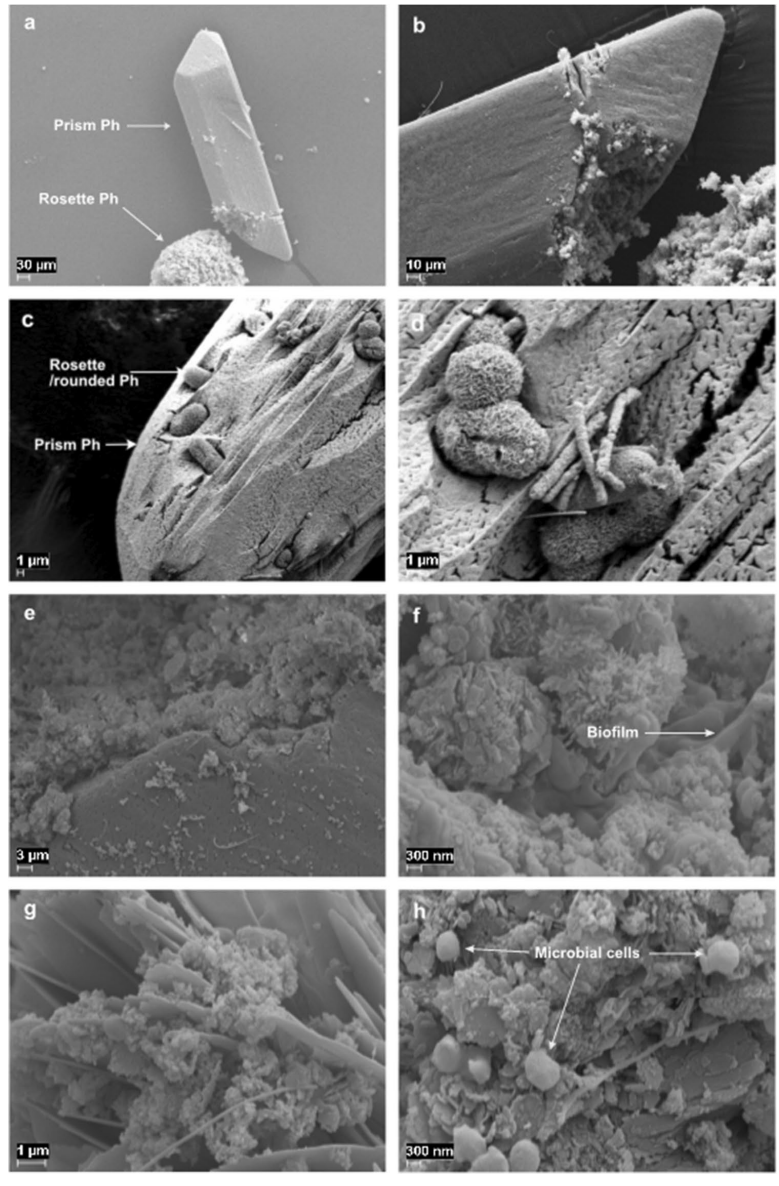

Fig. 7 SEM images of the cultures supplemented with zeolite. Images **a**–**d** were obtained from acetate-degrading cultures, while images **e**–**h** were obtained from propionate-degrading cultures

pH (7.3) prevailing at culture initiation. While the optimal pH for these enrichment cultures remains undetermined, an optimum range around 7.9–8.2 would align with conditions typically found in high-ammonia biogas processes (Westerholm et al. 2015, 2020; Fischer et al. 2019), from where the inoculum cultures used in these experiments were taken. Notably, higher pH increased the ammonia level in the medium, which commonly lowers overall microbial activity (Yenigün and Demirel 2013). However, performance studies conducted with the main SAOB used in the present work have shown that even at elevated ammonia levels up to 1.8 g L^{-1} (at pH 8.1 and 38 °C) methane formation is not impeded, but rather accelerated (Westerholm et al. 2019). Moreover, previous studies have found that the main SAOB

in this study, *S. schinkii*, cannot grow as pure culture at pH 8.5 (Westerholm et al. 2010). However, it has been shown that the growth parameters for organisms in pure culture can differ from those in co-culture (Hanly et al. 2012) and that formation of flocs (where these syntrophic microorganisms were found in higher abundance) can also provide protection against adverse conditions such as alkaline pH (Charles et al. 2017) and can increase tolerance to inhibitors (Westman et al. 2014).

The biphasic propionate degradation wherein acetate accumulated and underwent oxidation prior to ultimate degradation of propionate observed in P1 aligns with a previous study of the enriched mesophilic SPOB community used in the present study (Weng et al. 2024). In that study, thermodynamic calculations indicated low impact by acetate levels on the propionate degradation. Similarly, our results showed that propionate degradation by the SPO enrichment culture (initiated at 3.5 g L^{-1} propionate) was not impaired by acetate levels at or below 2 g L^{-1} (Fig. S7). An alternative explanation is that the onset of an accelerated phase of acetate degradation altered the environmental conditions, resulting in a rise in H_2 levels and in pH, and thus in ammonia levels (Fig. S3b, Fig. S4 and Fig. S5), temporarily impeding SPOB activity. Notably, a similar biphasic propionate degradation was not observed in P2, possibly because propionate was degraded faster and already completely consumed at the point of initiation of acetate degradation. However, in the control and the graphene batches in P2, the relatively slower degradation rate of propionate left approximately 0.2–0.3 g L^{-1} of propionate in the cultures when syntrophic acetate oxidation began. Consequently, this minor amount of propionate was not degraded until acetate was depleted, which happened after about 140 days of incubation (Fig. 2). Another interesting finding of the present study was that, unlike the acetate cultures, P2 did not show a shorter lag phase in any of the batches in comparison with P1. Syntrophic propionate oxidation has a notoriously long lag phase due to its energetically unfavourable reactions (Imachi et al. 2000, 2002). Metagenomic studies have indicated that, like several other SPOB (Kato et al. 2009; Hidalgo-Ahumada et al. 2018), '*Ca* S. ammoniitolerans' connects the first step of endergonic propionate activation with the last step of exergonic acetyl-CoA deactivation (Singh et al. 2021). This can explain the long lag phase in initial stages of propionate degradation and after a starvation period, i.e. at the beginning of P1 and P2.

In A1 and P1, methane yield from conversion of acetate or propionate corresponded to 88–100% of the expected methane production (Fig. S8, Fig. S9) based on the stoichiometry of the oxidation reactions (Table S3). This yield range is in line with values reported in previous studies on syntrophic propionate and acetate oxidation under high-ammonia and thermophilic conditions (Singh et al. 2023) and on syntrophic acetate oxidation under high-ammonia and mesophilic conditions (Westerholm et al. 2019). However, the significantly lower levels obtained in A2 and P2 (72–80% of expected methane production; Fig. S8, Fig. S9) given the existing biomass are puzzling. Further research into the carbon and energy balance of catabolic and anabolic reactions is required to explain this finding.

Additives affected microbial colonisation, floc formation and propionate and acetate degradation rates

The acetate and propionate enrichment cultures revealed some similar and some diverging impacts of the different additives on the degradation rate. Zeolite and iron oxide had positive effects on the degradation rate by the SAO (A2) and SPO enrichment cultures (P2). These were the two fastest batches to degrade acetate in both A2 and P2 (Figs. 1b and 2b), while during P1, both treatments promoted faster degradation of acetate, preventing these cultures from reaching a 1:1 ratio between propionate degradation and acetate formation, unlike in the control batches and particularly the graphene batches (Fig. S6). These results are in agreement with previous reports of positive effects of addition of magnetite ($Fe(II,III)_3O_4$) (Viggi et al. 2014; Wang et al. 2018a, b) and zeolites (Milán et al. 2001; Tada et al. 2005) on VFA degradation in anaerobic processes under high-ammonia conditions. The rates of acetate and propionate degradation observed in the present study were comparable to those in other studies degrading complex materials. For instance, in a previous study using sludge from a wastewater treatment facility as inoculum and an ammonia level of 5 g L^{-1} NH_4Cl, cultures with iron oxide nanoparticles had acetate degradation rates of approximately 0.075 g L^{-1} day^{-1} (Zhuang et al. 2018), while in the present study the rate in iron-oxide-amended cultures was 0.098 g L^{-1} day^{-1} for A2. Another study investigating the impact of iron oxide nanoparticles on degradation of propionate under low-ammonia conditions (0.5 g L^{-1} NH_4Cl) using an inoculum from waste-activated sludge observed propionate degradation rates of approximately 0.029 g L^{-1} day^{-1} after a second propionate feeding (Viggi et al. 2014). In the present study, the iron oxide-amended batches degraded propionate at a rate of 0.130 g L^{-1} day^{-1} after a second propionate feeding (P2). Overall, our results indicated higher rates of propionate and acetate degradation in enriched syntrophic cultures supplemented with iron oxide nanoparticles than in sludge inoculum supplemented with these acids in previous studies. These differences could be related to promotion of syntrophic interactions in the enrichment cultures by the iron oxide nanoparticles, whereas in more complex cultures this material may influence other microbial interactions than the syntrophic cooperation (Sarker and Nikhil 2023). Regarding

the positive effect of zeolite on acetate (A2) and propionate (P2) degradation demonstrated in the present study, some studies degrading complex substrates (food waste) have been performed under ammonia levels around 0.3 g NH_3 L^{-1}, at which some ammonia stress can be observed (Cardona et al. 2021). However, little information is available on acetate and propionate degradation by highly enriched syntrophic cultures, highlighting the novel value of our study.

Conductive additives have been suggested to enable DIET between microorganisms lacking e-pili or multiheme c-type cytochromes (Liu et al. 2012, 2015), which could be another possible explanation for the faster degradation rate in the iron oxide cultures in A2 and P2. The extensive colonisation of iron oxide nanoparticles (Fig. 5a–c), zeolites (Fig. 7e, h) and precipitates in these cultures suggests that the microbial community benefited from their presence as support structures. These additives may facilitate establishment of interspecies connections and short-circuit the electron transfer, either through DIET or through diffusion of intermediary compounds such as formate and H_2, or possibly through co-occurrence of both processes (Thiele and Zeikus 1988; Dolfing 2013). Zeolites may have contributed to the enhanced degradation rates observed in A2 and P2, potentially due to their content of loosely bound surface cations (e.g. Ca^{2+} and Mg^{2+}). When exchanged with other ions present in the solution (Pabalan and Bertetti 2001), such as NH_4^+, these cations could have promoted the capability for acid degradation in the culture. This could have alleviated the ammonia stress (Wang and Peng 2010), while the slightly elevated Ca^{2+} and Mg^{2+} concentrations within the solution could also have stimulated microbial activity (Wang et al. 2023). For instance, Ca^{2+} is an important regulator that can influence bacterial cell structure, differentiation and gene expression (Smith 1995) and biofilm formation, regulating the diffusion of fluids, inorganic and organic compounds and toxic molecules (Keren-Paz and Kolodkin-Gal 2020). Furthermore, Mg^{2+} is a cofactor for a wide range of enzymes that stabilise nucleic acids and ribosomes, for ATPases and for maintaining cell integrity (Smith and Maguire 1998).

The formation of pyrites (FeS_2, cubic) in the iron oxide-supplemented acetate- and propionate-degrading cultures (Fig. 5d) indicated that the additional available iron altered the chemical conditions for the syntrophic communities. The basal medium used in the present study had redox potential of − 330 mV and initial pH of 7.3, which is within the solid stability field of the Fe-S system of FeS and possibly FeS_2 (Lyon 2010). The basal medium also had a Fe concentration of 10^{-6} M and S concentration of 10^{-3} M, and was thus already saturated with respect to formation of iron sulphides, such as cubic FeS_2 and tetragonal layered FeS. Hence, in the iron oxide-supplemented cultures, the increase in pH to 7.9–8.2 during growth most likely promoted formation of cubic FeS_2. The initial pH in the cultures could have enabled formation of tetragonal layered FeS, but only cubic FeS_2 was observed in the samples. Nevertheless, FeS is within the stability field and may therefore also be present in the samples. To scan the solid phase for secondary minerals, further SEM–EDS mapping or X-ray diffraction analysis would be required. Vivianite $\left(Fe_3^{2+}\left(PO_4\right)_2 \cdot 8H_2O\right)$ precipitates were also formed in the iron oxide-supplemented cultures. Formation of this precipitate when phosphates and $Fe(II,III)_3O_4$ are present in the medium has been reported previously (Hao et al. 2022; Prot et al. 2022). Our element analyses also demonstrated formation of an extensive amount of phosphate precipitates in the zeolite-supplemented cultures. The impact of formation of these precipitates on acid degradation rate is still unclear and further research is required, but several studies have shown that phosphates in solution can reduce methanogenic activity under low-ammonia conditions and hinder consumption of acids such as acetate, propionate and butyrate (Paulo et al. 2005; Lackner et al. 2020). However, it was not possible to assess the effect of phosphate dissolution or precipitation on acid consumption and methanogenesis in the present study.

Interestingly, graphene had a positive effect on propionate degradation rate in the SPO enrichment cultures, but not on acetate degradation in the SAO enrichment cultures. The positive effect in P1 and P2 could have the same explanation as suggested for the effect of iron oxide nanoparticles in A2 and P2, as graphene itself is a conductive material that can permit DIET (Florentino et al. 2019; Zhou et al. 2022). It has even been shown that addition of graphene to a biogas process degrading food waste can promote e.g. lower propionate accumulation and shorter culture lag phase (Capson-Tojo et al. 2018). However, the impaired acid degradation in both A1 and A2 is intriguing and supports the higher acetate accumulation resulting from propionate degradation in P1, in the graphene-amended batches. The reason for this negative impact is not clear, but it has been suggested that carbon nanoparticles can interfere with cell membrane integrity, thereby compromising cell viability and metabolic capacity (Akhavan and Ghaderi 2010; Zhou and Gao 2014). Previous studies in the degradation of sludge fed with acetate had already shown that acetate degradation was not positively affected by addition of granular activated carbon (Xu et al. 2018). Moreover, the colonies of microorganisms in between the graphene grains were not as extensive as in the iron oxide- and zeolite-amended cultures (Fig. 7b–d and f–h), where the entire surface of the additives was covered by microbial colonies, and phosphate precipitates were not formed in these samples. These results indicate that graphene addition promoted biofilm formation to a lesser extent than the other additives. This can help explain the lower rate of acid degradation in the SAO enrichment cultures and the accumulation of acetate in P1 in the graphene-amended cultures compared with the iron oxide and zeolite cultures, since syntrophic metabolism relies on physical proximity

of the acid oxidisers and their partner methanogen (Cord-Ruwisch et al. 1998; Felchner-Zwirello et al. 2013). However, the apparent similar rate of acetate degradation after peaking acetate values during P1 and P2 remains enigmatic.

Higher abundance of SPOB, SAOB and methanogens in flocculating communities reflects high importance of cell proximity for syntrophic activities

The relative microbial abundance profile in the SAO enrichment cultures indicated that over half of the microbial community at the end of A1 comprised members of the genus *Alkaliphilus*. This genus has previously been suggested to include potential ammonia-tolerant SAOB (Mosbaek et al. 2016; Westerholm et al. 2018), but this has not yet been proven. It should be noted that *Alkaliphilus* sp. has the ability to grow on several substrates, such as the yeast extract added to the medium in the present study (Takai et al. 2001; Zhilina et al. 2009). In contrast, *Alkaliphilus* sp. was not detected at high relative abundance in our SPO enrichment cultures, except for the planktonic iron oxide communities (Fig. 3). Members of *Alkaliphilus* are alkaliphilic and have a pH optimum between 7.5 and 10 (Takai et al. 2001; Cao et al. 2003; Fisher et al. 2008), which coincides with the higher pH in the SAO cultures, while the lower pH in the SPO cultures would be less favourable for the *Alkaliphilus* member present in the SAO cultures. *Syntrophaceticus schinkii* was present in high relative abundance in both the SAO and SPO enrichment cultures. This species is a known SAOB (Westerholm et al. 2010), suggesting a significant role as the primary acetate-degrading species in the SPO enrichment cultures and very likely also in the SAO enrichment cultures. The qPCR results showed that *S. schinkii* was present in higher abundance in flocculating communities than in planktonic communities across all SPO and SAO enrichment cultures, alongside their partner methanogen (this latter with the exception of the acetate-fed, iron oxide- and graphene-amended batches). This strongly indicates that these syntrophic microorganisms strive for physical proximity to facilitate their syntrophic metabolism. Interestingly, zeolite addition seemed to promote recruitment of *S. schinkii* in both the acetate- and propionate-fed batches, which had significantly higher levels of this SAOB than the control ($p < 0.05$), while graphene seemed to promote its presence in the propionate-fed batches ($p < 0.03$). As found in a previous SAO enrichment study (Westerholm et al. 2019), another known SAOB, *T. acetatoxydans*, was present at considerably lower relative abundance than *S. schinkii*, in particular in the flocculating communities. In fact, its relative abundance in the acetate-degrading enrichments was even lower than 3% in all batches. This suggests that *T. acetatoxydans* was a secondary SAOB in both the acetate- and propionate-degrading enrichment cultures and did not play such an important role in the flocculating communities.

As with the SPOB, the high relative abundance of '*Ca* S. ammoniitolerans' in the propionate-fed batches (and its absence in the SAO enrichment cultures) indicated that it was the main SPOB in the propionate-degrading enrichment cultures. This species has previously been identified as a candidate SPOB in the reactors from which the inoculum used in the present study originated (Singh et al. 2021). The qPCR results confirmed that this SPOB was present in significantly higher abundance in flocculating communities than in planktonic communities ($p < 0.02$), further stressing the importance of these syntrophic microorganisms living in close proximity to complete their metabolism. The qPCR results indicated that iron oxide addition promoted recruitment of this SPOB to flocculating communities, where it displayed significantly higher levels than in control batches ($p = 0.04$). Another relevant finding is presence of the genus *Acetomicrobium* in the planktonic samples. Activity of members of this genus has been identified previously in the mesophilic community used in the present study (Weng et al. 2024) as well in acetate- and propionate-fed reactors under thermophilic conditions (Singh et al. 2023). It has been hypothesised that this species might be involved in formate utilisation or conduct syntrophic acetate oxidation, but the species is also described as capable of growing on a broad spectrum of substrates, including yeast extract and cysteine (Menes and Muxí 2002; Hania et al. 2016) which is present in the medium used in the present study.

As found in several previous microbial studies with syntrophic cultures and high-ammonia conditions (Westerholm et al. 2010, 2019; Maus et al. 2015; Manzoor et al. 2016), a member of the genus *Methanoculleus* was the main partner methanogen for the syntrophic bacteria. *Methanoculleus bourgensis* has previously been found to be the partner methanogen in co-culture with *S. schinkii* (Westerholm et al. 2019), whereas the partner methanogen to the SPOB and SAOB in enrichment cultures in the present study has previously been identified to be a novel *Methanoculleus* species, with the provisional name '*Candidatus* Methanoculleus ammoniitolerans' (Weng et al. 2024). The qPCR results showed that hydrogenotrophic methanogens were also present in significantly higher abundance in the flocculating communities than in the planktonic communities ($p < 0.01$), with the exception of the SAO enrichment cultures treated with iron oxide ($p > 0.8$) and graphene. It is worth pointing out that no other group of methanogens was present in relative abundance of at least 1%, in agreement with findings in other studies conducted on anaerobic digestion under high-ammonia conditions (Schnürer and Nordberg 2008; Fotidis et al. 2014; Westerholm et al. 2015). To sum up, a significant increase in abundance of certain syntrophic microorganisms within flocculating communities compared with planktonic communities was observed. Specifically, the SPOB '*Ca* S. ammoniitolerans' and SAOB *S. schinkii* showed higher abundance within flocculating communities in all propionate-fed enrichment cultures ($p < 0.03$), with iron oxide

addition significantly promoting the recruitment of the SPOB ($p = 0.04$), and graphene and zeolite addition significantly promoting the recruitment of the SAOB to these communities ($p < 0.03$). Furthermore, *S. schinkii* was present in higher abundance within flocculating communities in all acetate-fed enrichments. Additionally, there was notably higher abundance of *Methanoculleus* sp. in all propionate- and acetate-fed enrichment cultures ($p < 0.01$) except for the acetate-fed enrichments amended with iron oxide or graphene. These results are in accordance with previous studies (Cheng and Call 2016; Wang et al. 2019) and suggest that the impact of additives may derive not only from promotion of syntrophic activity, but also from number of syntrophic microorganisms recruited in flocs.

To conclude, addition of iron oxide nanoparticles and zeolite enhanced the rate of both acetate and propionate degradation, following a second acid addition. Both additives also promoted microbial colonisation of their surfaces, enabling close proximity between microbial cells that likely facilitated faster acid consumption. Addition of graphene impaired the rate of acetate degradation and did not promote microbial colonisation of its surface but promoted propionate degradation. Microbial community composition and abundance of syntrophic bacteria and associated methanogens differed markedly between flocs and planktonic samples. The main genus present in acetate cultures was *Alkaliphilus* sp., followed by the SAOB *S. schinkii*, which was found in higher abundance in flocs than in planktonic communities, except in acetate-fed, iron oxide- and graphene-amended cultures. In the propionate cultures, the candidate SPOB was identified as '*Ca* S. ammoniitolerans', while the SAOB was *S. schinkii*. Both these species were present in significantly higher abundance in flocs than in planktonic communities in all propionate-degrading cultures. In both acetate and propionate cultures, the partner methanogen was '*Ca*. *Methanoculleus* ammoniitolerans', which was present in significantly higher abundance in flocs than in planktonic communities in all propionate cultures and in the acetate control cultures. Furthermore, the cultivation experiments with different acetate levels revealed that the acetate concentration per se had minimal influence on propionate degradation. Nevertheless, the outcome implied that the two syntrophic bacteria (the SPOB and the SAOB) encountered challenges in maintaining simultaneous activity.

Novel approaches that enhance SAOB and SPOB abundance and activity in biogas reactors will result in better reactor performance in high-ammonia biogas processes, by preventing VFA accumulation and lowered methane yield, and a risk of complete system failure. Addition of conductive and non-conductive additives such as the ones used in this study can stimulate syntrophic metabolism by aiding floc formation, precipitating or removing compounds known to inhibit different steps of the anaerobic digestion process, or by providing nutrients which are required by these microorganisms to fulfil their metabolisms. While graphene may not be an additive to consider for an industrial-scale biogas digester due to its cost, iron oxide particles and zeolites are inexpensive, readily available additives with good prospects of being applied in industrial-scale biogas digesters operating under high-ammonia conditions. Further experiments focusing on the suitability of such additives for different types of reactors and operating conditions (type of substrate, temperature, pH, ammonia levels, etc.) and on the optimisation of concentration of additives at small-scales (to allow validation of laboratory-scale results and troubleshooting of possibly emerging operational problems) should then be conducted before industrial implementation. It is therefore of critical importance for biogas production processes to address ammonia inhibition through identification and inclusion of suitable additives, evaluation of their feasibility of use and optimisation from a functional, economic and environmental standpoint.

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Data availability The raw sequencing data obtained in this study are available at NCBI under BioProject 'Impact of supportive materials on syntrophic propionate and acetate enrichments under high-ammonia' (PRJNA1069746).

Declarations

Ethics approval This study did not contain any analyses involving human participants or animals.

Conflict of interest The authors declare no competing interests.

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Supplementary Information (SI)

Applied Microbiology and Biotechnology

Impact of additives on syntrophic propionate and acetate enrichments under high-ammonia conditions

Eduardo Pinela[1], Anna Schnürer[1], Anna Neubeck[2], Jan Moestedt[1,3,4], Maria Westerholm[1, *]

[1]Department of Molecular Sciences, Swedish University of Agricultural Sciences, Uppsala, SE-750 07, Sweden

[2]Department of Earth Sciences, Uppsala University, Uppsala SE-752 36, Sweden

[3]Department of Biogas R & D, Tekniska verken i Linköping AB (publ.), Box 1500, Linköping, SE-581 15, Sweden

[4]Department of Thematic Studies - Environmental Change, Linköping University, Linköping SE-581 83, Sweden

***Correspondence:**
Maria Westerholm
Maria.Westerholm@slu.se ORCID: 0000-0003-2150-8762

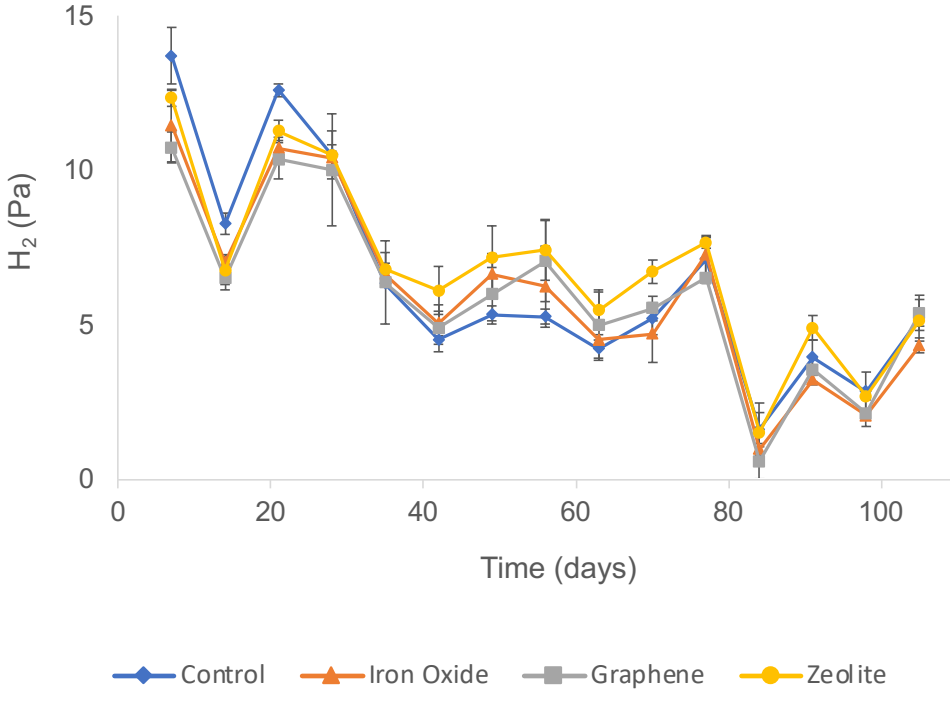

Fig. S1 Variation in H_2 partial pressure (Pa) during the first round of acetate degradation (A1) in syntrophic acetate-oxidising (SAO) enrichment cultures

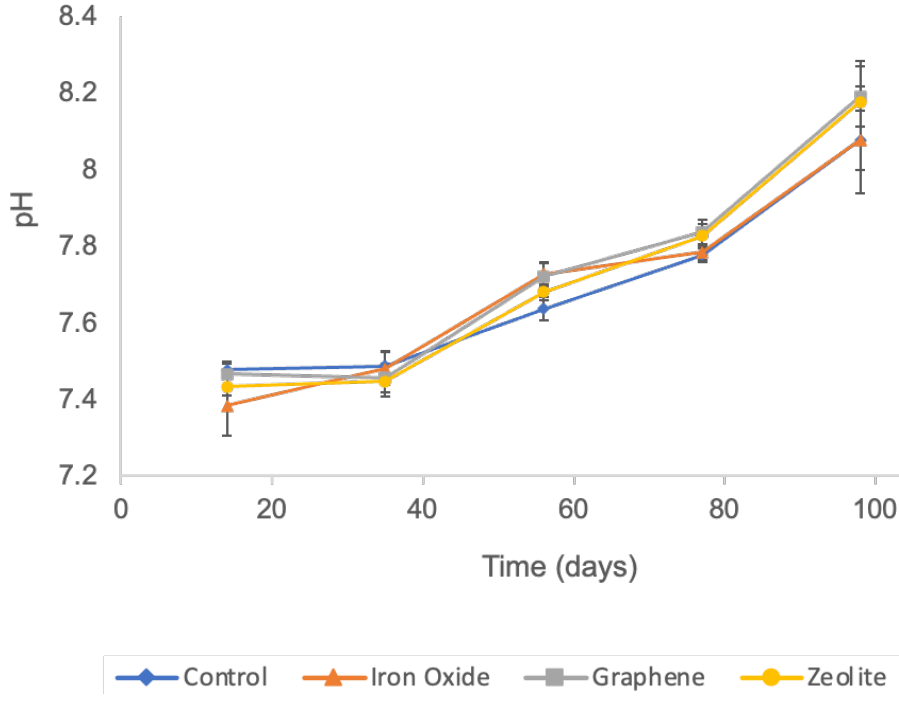

Fig. S2 Variation in pH during the first round of acetate degradation (A1) in syntrophic acetate-oxidising (SAO) enrichment cultures

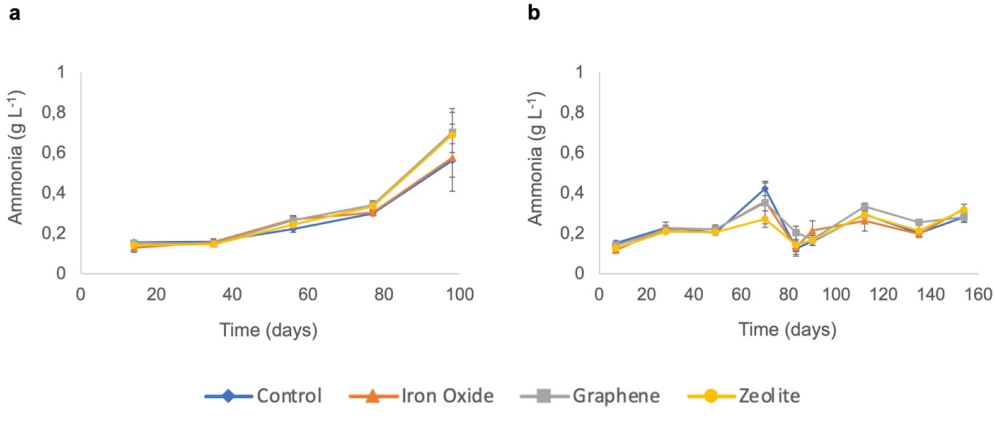

Fig. S3 Variation in ammonia concentration during the first round of (**a**) acetate degradation (A1) in syntrophic acetate-oxidising (SAO) enrichment cultures and (**b**) propionate degradation (P1) in syntrophic propionate-oxidising (SPO) enrichment cultures. Values calculated by entering pH values into the equation in Table S4

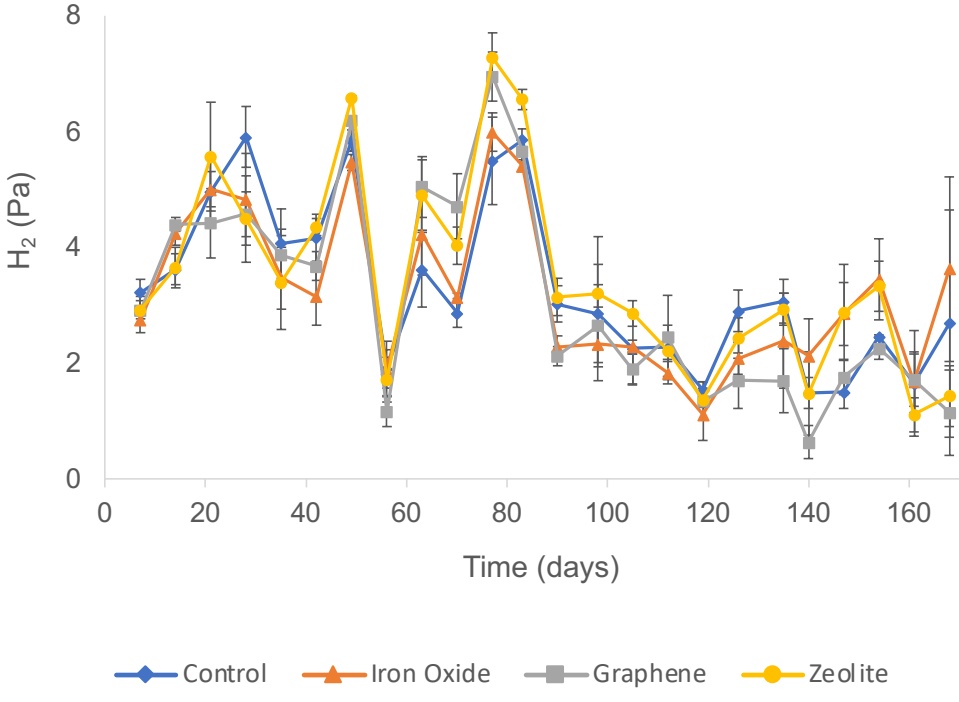

Fig. S4 Variation in H_2 partial pressure (Pa) during the first round of propionate degradation (P1) in syntrophic propionate-oxidising (SPO) enrichment cultures

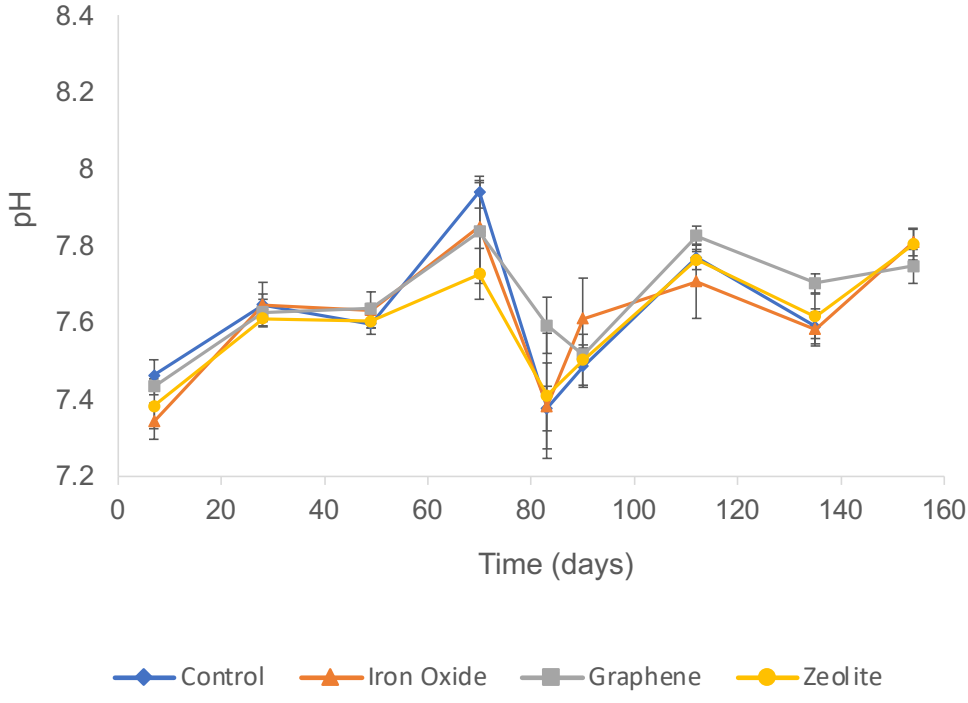

Fig. S5 Variation in pH during the first round of propionate degradation (P1) in syntrophic propionate-oxidising (SPO) enrichment cultures

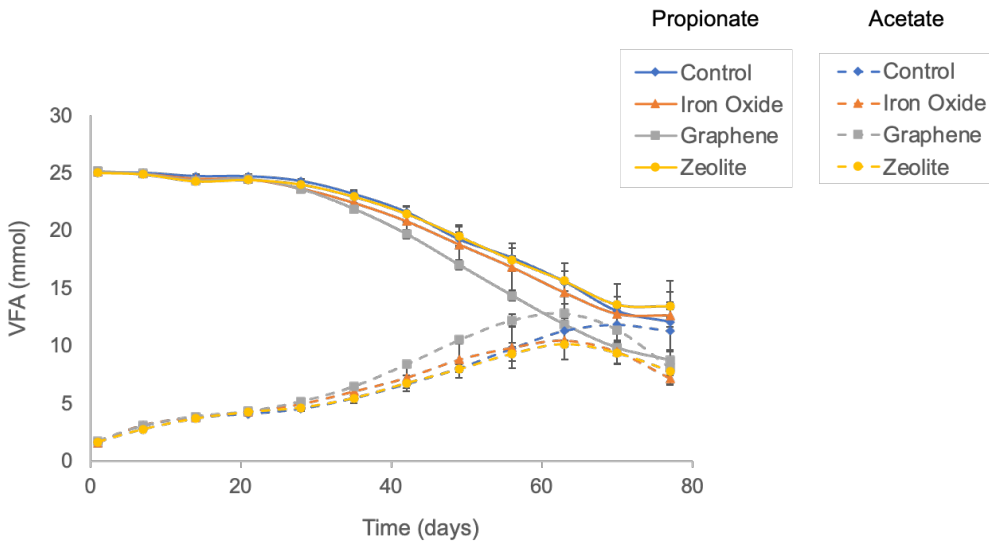

Fig. S6 Molar conversion of propionate into acetate up to peak acetate level (between days 63-70) during the first round of propionate degradation (P1) in syntrophic propionate-oxidising (SPO) enrichment cultures

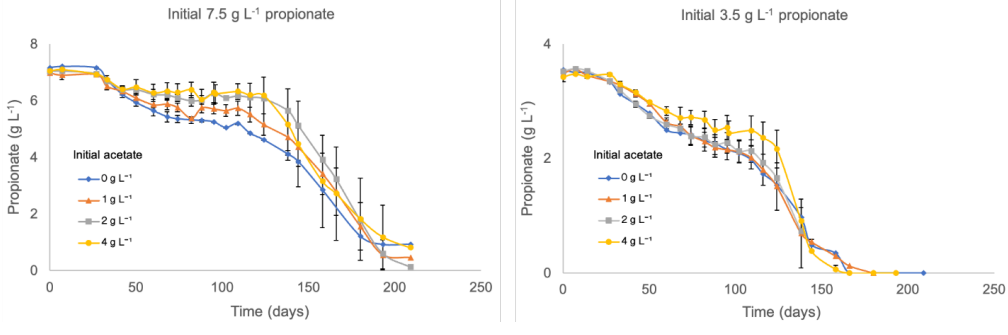

Fig. S7 Impact of acetate (initial concentration 0, 1, 2 or 4 g L^{-1}) on propionate degradation (initial concentration of 7.5 or 3.5 g L^{-1}). Note the different scale on the y-axes

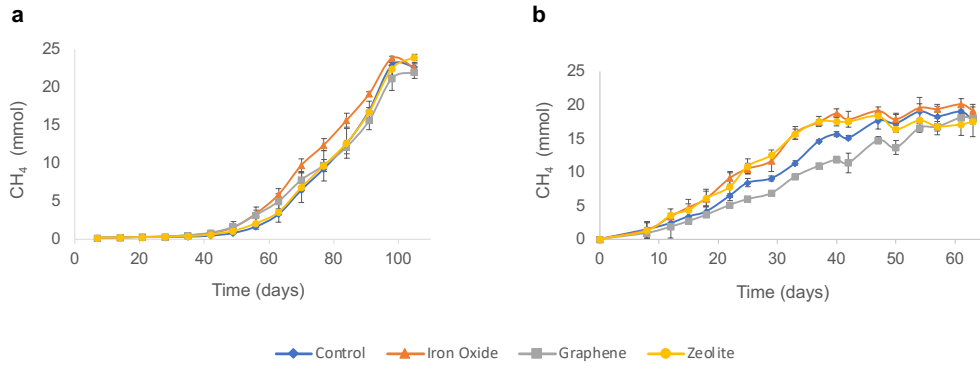

Fig. S8 Moles of methane produced in the acetate-fed batches during (**a**) the first (A1) and (**b**) the second (A2) round of acetate degradation

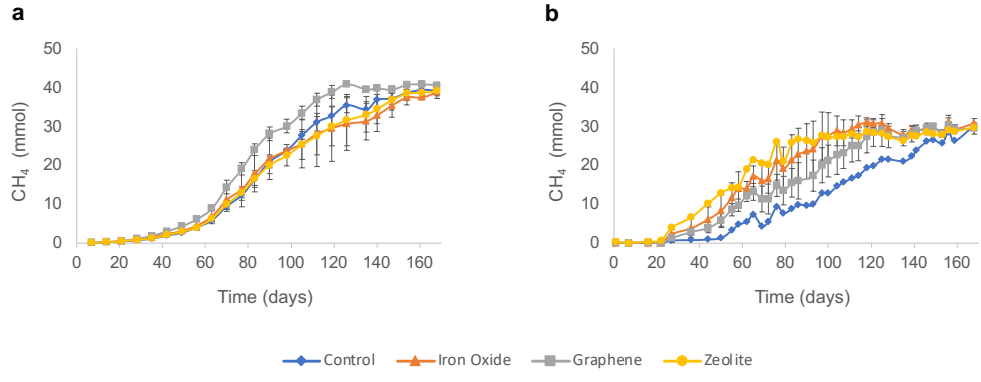

Fig. S9 Moles of methane produced in the propionate-fed batches during (**a**) the first (P1) and (**b**) the second (P2) round of propionate degradation. Averages for the control and zeolite batches during P2 were calculated using duplicates

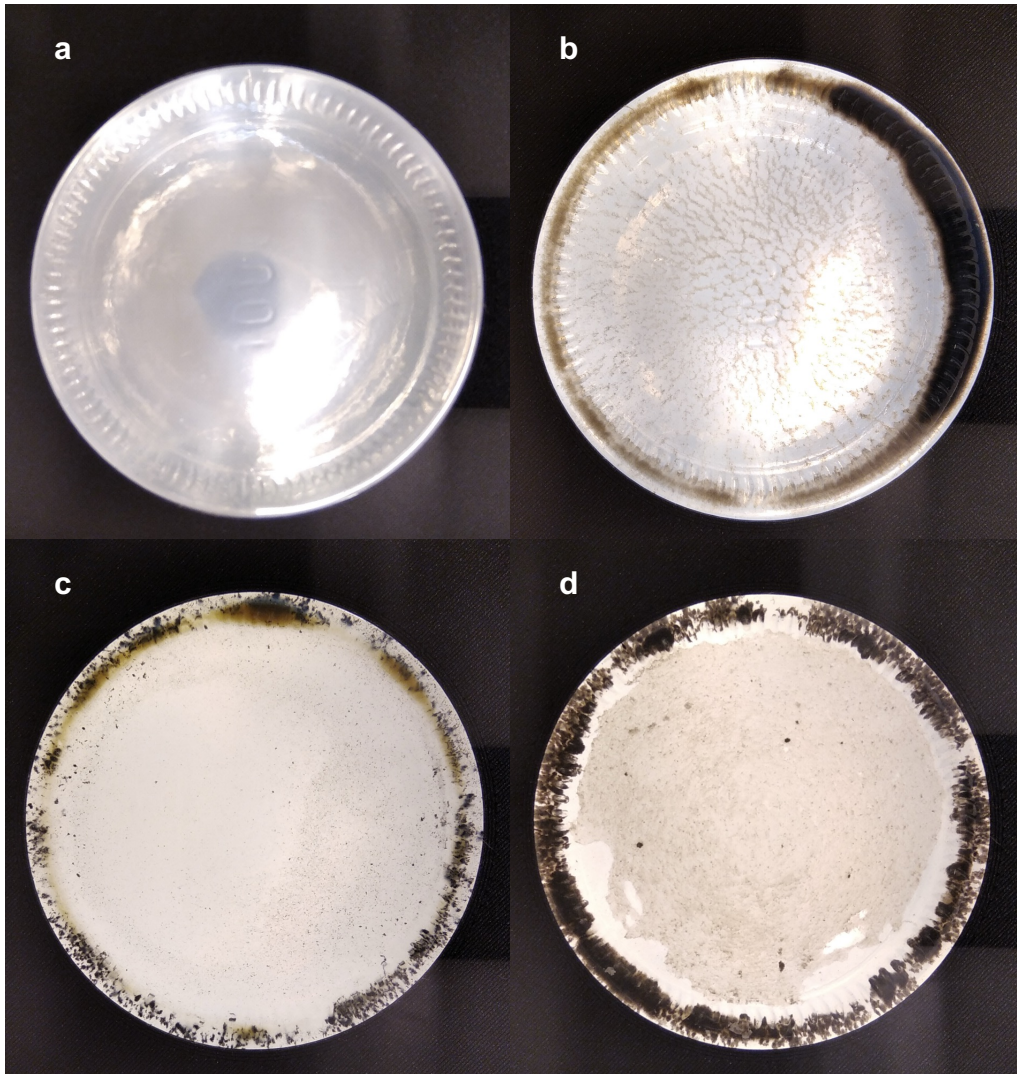

Fig. S10 Biomass observable to the naked eye at the start of the first round of (**a**) acetate (A1) and (**b**) propionate (P1) degradation, and at the end of the second round of (**c**) acetate (A2) and (**d**) propionate (P2) degradation in the syntrophic acetate-oxidising (SAO) and syntrophic propionate-oxidising (SPO) enrichment cultures, respectively. Images (**a**) and (**b**) were obtained at day 1 and day 3 of A1 and P1, respectively, while images (**c**) and (**d**) were obtained at day 104 and day 168 of A2 and P2, respectively

Table S1 Experimental set-up of batch trials investigating the impact of presence of acetate without addition of supportive materials on propionate degradation. All batch sets were run in triplicate

Batch set	Propionate mM (g L^{-1})	Acetate mM (g L^{-1})
1	47 (3.5)	0
2	47 (3.5)	14 (0.8)
3	47 (3.5)	29 (1.7)
4	47 (3.5)	61 (3.6)
5	95 (7.1)	0
6	95 (7.1)	14 (0.8)
7	95 (7.1)	29 (1.7)
8	95 (7.1)	61 (3.6)

Table S2 Values used in calculation of acid degradation rates during the first (P1) and second (P2) rounds of propionate degradation and during the first (A1) and second (A2) rounds of acetate degradation

Round	Acid	Batch set	Acid level at first day of linear degradation phase (g L^{-1})	Acid level at last day of linear degradation phase (g L^{-1})	Number of days in between two acid levels	Rate of degradation (g L^{-1} day^{-1})
First	Propionate	Control 1	3.279	1.821	35	0.042
		Control 2	3.255	1.892	35	0.039
		Control 3	3.219	1.815	35	0.040
		Iron Oxide 1	3.049	1.389	35	0.047
		Iron Oxide 2	3.146	2.061	35	0.031
		Iron Oxide 3	3.248	1.984	35	0.036
		Graphene 1	3.073	1.348	35	0.049
		Graphene 2	3.045	1.443	35	0.046
		Graphene 3	3.107	1.385	35	0.049
		Zeolite 1	3.281	2.027	35	0.036
		Zeolite 2	3.253	1.903	35	0.039
		Zeolite 3	3.139	1.845	35	0.037
Second	Propionate	Control 1	3.265	1.914	14	0.096
		Control 2	3.158	1.834	14	0.095
		Control 3	N/A	N/A	N/A	N/A
		Iron Oxide 1	1.803	0.134	14	0.119
		Iron Oxide 2	3.164	1.409	14	0.125

		Iron Oxide 3	2.760	0.739	14	0.144
		Graphene 1	2.906	1.599	14	0.093
		Graphene 2	3.165	1.781	14	0.099
		Graphene 3	2.633	1.118	14	0.108
		Zeolite 1	N/A	N/A	N/A	N/A
		Zeolite 2	1.524	0.200	14	0.095
		Zeolite 3	1.865	0.157	14	0.122
		Control 1	2.484	0.744	21	0.083
		Control 2	2.502	0.748	21	0.084
		Control 3	2.544	0.811	21	0.083
		Iron Oxide 1	2.115	0.333	21	0.085
		Iron Oxide 2	1.997	0.252	21	0.083
First	Acetate	Iron Oxide 3	2.179	0.447	21	0.082
		Graphene 1	2.419	0.748	21	0.080
		Graphene 2	2.062	0.727	21	0.064
		Graphene 3	2.282	1.094	21	0.057
		Zeolite 1	2.638	1.032	21	0.077
		Zeolite 2	2.213	0.396	21	0.087
		Zeolite 3	2.529	0.827	21	0.081
		Control 1	2.847	0.924	25	0.077
		Control 2	2.891	0.890	25	0.080
		Control 3	3.181	1.002	25	0.087
		Iron Oxide 1	2.798	0.338	25	0.098
		Iron Oxide 2	2.799	0.455	25	0.094
Second	Acetate	Iron Oxide 3	2.878	0.330	25	0.102
		Graphene 1	2.899	1.433	25	0.059
		Graphene 2	2.540	1.209	25	0.053
		Graphene 3	3.271	1.420	25	0.074
		Zeolite 1	2.697	0.361	25	0.093
		Zeolite 2	3.238	0.198	25	0.122
		Zeolite 3	2.850	0.100	25	0.110

Table S3 Carbon balance calculations during the first (P1) and second (P2) rounds of propionate degradation and during the first (A1) and second (A2) rounds of acetate degradation. Theoretical moles of CH_4 were calculated using conversion coefficients of 1.75 and 1 from propionate and acetate, respectively

Round	Acid	Batch set	Starting moles of acid (mmol)	Theoretical moles of CH4 (mmol)	Moles of CH4 obtained (mmol)	Yield (%)
First	Propionate	Control 1	25	43.75	37.56	85.85
		Control 2	25	43.75	39.04	89.23
		Control 3	25	43.75	40.81	93.27
		Iron Oxide 1	25	43.75	39.63	90.59
		Iron Oxide 2	25	43.75	38.14	87.18
		Iron Oxide 3	25	43.75	38.67	88.39
		Graphene 1	25	43.75	40.90	93.49
		Graphene 2	25	43.75	40.25	92.01
		Graphene 3	25	43.75	40.57	92.74
		Zeolite 1	25	43.75	39.89	91.19
		Zeolite 2	25	43.75	41.03	93.78
		Zeolite 3	25	43.75	36.49	83.40
Second	Propionate	Control 1	25	43.75	34.83	79.61
		Control 2	25	43.75	28.65	65.47
		Control 3	25	43.75	27.79	63.53
		Iron Oxide 1	25	43.75	32.09	73.35
		Iron Oxide 2	25	43.75	29.69	67.87
		Iron Oxide 3	25	43.75	30.86	70.55
		Graphene 1	25	43.75	30.96	70.76
		Graphene 2	25	43.75	29.68	67.84
		Graphene 3	25	43.75	28.10	64.24
		Zeolite 1	25	43.75	24.96	57.06
		Zeolite 2	25	43.75	33.09	75.64
		Zeolite 3	25	43.75	31.51	72.02
First	Acetate	Control 1	25	25	22.53	90.12
		Control 2	25	25	23.26	93.06
		Control 3	25	25	22.14	88.55
		Iron Oxide 1	25	25	22.55	90.18
		Iron Oxide 2	25	25	23.37	93.50

		Iron Oxide 3	25	25	21.70	86.81
		Graphene 1	25	25	23.04	92.16
		Graphene 2	25	25	21.54	86.16
		Graphene 3	25	25	21.27	85.09
		Zeolite 1	25	25	24.31	97.23
		Zeolite 2	25	25	23.40	93.59
		Zeolite 3	25	25	24.04	96.14
Second	Acetate	Control 1	25	25	19.44	77.77
		Control 2	25	25	17.76	71.03
		Control 3	25	25	19.81	79.25
		Iron Oxide 1	25	25	19.42	77.70
		Iron Oxide 2	25	25	19.83	79.31
		Iron Oxide 3	25	25	21.02	84.06
		Graphene 1	25	25	18.17	72.68
		Graphene 2	25	25	18.49	73.95
		Graphene 3	25	25	17.77	71.07
		Zeolite 1	25	25	15.33	61.32
		Zeolite 2	25	25	18.66	74.65
		Zeolite 3	25	25	17.30	69.19

Table S4 Equation and variables used in calculation of ammonia values

Temperature (K)	273.15 + Temperature (°C)
pKa	$0.09018 + \left(\frac{2729.92}{\text{Temperature (K)}}\right)$
NH_3 (g L^{-1})	$\frac{NH_4\ (g\ L^{-1})}{\left(1+10^{(pKa-pH)}\right)}$

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2025:43

Biogas production converts biodegradable waste into biogas and digestate, but faces challenges like ammonia inhibition. Ammonia inhibits microbial activity, making ammonia-tolerant syntrophic microorganisms crucial for degrading propionate and acetate into biogas. This thesis investigates strategies like adding materials, iron and sulfur compounds, and adjusting ammonia levels, zeolites, and temperature to improve microbial performance. Results show improved acid degradation and methane production, though success depends on managing ammonia, temperature, and microbial community, enhancing the resilience of biogas processes under high-ammonia conditions.

Eduardo Pinela received his graduate education at the Department of Molecular Sciences, SLU, Uppsala. He received his M.Sc. in Applied Biotechnology at Uppsala University and his B.Sc. in Biology at Lisbon University.

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