Bioresource Technology 247 (2018) 999–1014

Contents lists available at ScienceDirect

Bioresource Technology

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

Review

Inhibition of anaerobic digestion processes: Applications of molecular tools

Yamrot M. Amha[a], Muhammad Zohaib Anwar[b,c], Andrew Brower[d], Carsten S. Jacobsen[b,c], Lauren B. Stadler[d], Tara M. Webster[e], Adam L. Smith[a,*]

[a] Astani Department of Civil and Environmental Engineering, University of Southern California, 3620 South Vermont Avenue, Los Angeles, CA 90089, USA
[b] mBioInform ApS, Ole Maaloes Vej 3, 2200 Copenhagen N, Denmark
[c] Department of Environmental Sciences, Aarhus University, Frederiksborgvej, 399, 4000 Roskilde, Denmark
[d] Department of Civil and Environmental Engineering, Rice University, 6100 Main Street, TX 77005, USA
[e] Soil and Crop Sciences Section, Cornell University, 306 Tower Road, Ithaca, NY 14853, USA

A R T I C L E I N F O

Keywords:
Next generation sequencing
16S rRNA
Functional genes
Omics
Real-time monitoring

A B S T R A C T

Inhibition of anaerobic digestion (AD) due to perturbation caused by substrate composition and/or operating conditions can significantly reduce performance. Such perturbations could be limited by elucidating microbial community response to inhibitors and devising strategies to increase community resilience. To this end, advanced molecular methods are increasingly being applied to study the AD microbiome, a diverse community of microbial populations with complex interactions. This literature review of AD inhibition studies indicates that inhibitory concentrations are highly variable, likely stemming from differences in community structure or activity profile and previous exposure to inhibitors. More recent molecular methods such as 'omics' tools, substrate mapping, and real-time sequencing are helping to unravel the complexity of AD inhibition by elucidating physiological and ecological significance of key microbial populations. The AD community must strive towards developing predictive abilities to avoid system failure (e.g., real-time tracking of an indicator species) to improve resilience of AD systems.

1. Introduction

Anaerobic digestion (AD) is a waste management biotechnology that employs a diverse consortium of microorganisms to convert organics into methane-rich biogas. AD reduces organic waste landfilling and recovers energy via cogeneration of produced biogas. Given available organic waste feedstocks worldwide, biogas recovered from AD has the potential to provide a quarter of the world's natural gas demand and 6% of primary energy demand (Guo et al., 2015b). Despite the potential for AD to significantly contribute to our energy portfolio, biogas remains an underutilized resource with only 47–95 billion kWh of electricity generated from biogas in 2012, contributing just 0.2–0.4% of global electricity production (De Vrieze and Verstraete, 2016; Enerdata, 2015). Improving AD implementation requires that we overcome existing technological challenges that limit its widespread adoption (e.g., slow startup, low energy recovery, and inhibition).

AD is an engineered ecosystem where organic waste degradation takes place via a complex cascade of microbially-driven reactions including hydrolysis, fermentation (i.e., acidogenesis and acetogenesis), and methanogenesis. Energy recovery thus relies on functional activity of a wide range of Bacteria and Archaea, making it necessary to configure and operate AD systems such that conditions are conducive for diverse microbial populations. High complexity within the AD microbiome makes the process vulnerable to upset due to inhibition via accumulation of long chain fatty acids (LCFA), volatile fatty acid (VFA), free ammonia, and other compounds or unfavorable operating conditions, such as temperature and pH (Chen et al., 2014a). Although AD can recover energy from a wide range of organics (e.g., wastewater sludges; animal manure; food waste; and fats, oils, and grease (FOG)), feedstock variability leads to operational uncertainty which can reduce energy recovery or ultimately result in system failure. Therefore, significant effort has been placed on establishing inhibitory levels of specific compounds in AD, as summarized in Tables 1–4. However, inhibitory levels are strongly influenced by microbial community structure and activity. For example, functionally redundant populations within the AD microbiome can prevent upset by limiting accumulation of inhibitory intermediates. Thus, inoculum selection and temporal adaptation to inhibitors can prevent process failure (Silva et al., 2014; Silvestre et al., 2011), but we require a better understanding of the AD microbiome to accurately predict and prevent inhibition.

Recent advances in molecular methods have made it possible to study the structure, function, and interaction of increasingly complex

* Corresponding author.
E-mail address: smithada@usc.edu (A.L. Smith).

http://dx.doi.org/10.1016/j.biortech.2017.08.210
Received 8 July 2017; Received in revised form 30 August 2017; Accepted 31 August 2017
Available online 04 September 2017
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Table 1
Studies investigating volatile fatty acid (VFA) inhibition.

Reactor	Temp.	Feed	Performance impact	Microbial community analysis tool	Effect on microbial community	Reference
Full-scale	Mesophilic and thermophilic	Cow manure and food waste	● No inhibition detected even with high VFA (e.g., propionate concentrations of 8741 mg L^{-1})	ANAEROCHIP microarray and real-time qPCR	● *Methanothermobacter* dominated in thermophilic reactor with high VFA ● *Methanosarcina* increased in dominance with higher levels of VFAs	Franke-Whittle et al. (2014)
Batch	Thermophilic	^{13}C and ^{12}C labelled acetate	● High acetate fed reactor (8.2 g L^{-1}) showed linear acetate removal in the first 120 h (83% of the amended U-^{13}C)	Protein-SIP and metagenome	● *Methanosarcina*, *Methanoculleus*, and five subspecies of *Clostridia* (potential SAOBs) involved in recovery after inhibition ● Identified *Clostridia* groups contained the *fthfs* gene	Mosbæk et al. (2016)
Batch	Thermophilic	Sodium acetate	N/A	Isotope labeled substrate assays, protein-SIP, metagenomics	● Identified *Clostridia*, *Methanosarcina*, and *Methanoculleus* as being part of the recovery from high acetate conditions.	Mulat et al. (2014)
Batch	Mesophilic	Cellulose	● Critical acetate concentration was 25 mmol L^{-1} ● pH should be maintained at 7 to enhance cellulose hydrolysis rate	N/A	● N/A	Romsaiyud et al. (2009)
CSTR	Thermophilic	Sugar beet tailings	● Lower methane yield, delayed methane production rate, and propionic acid accumulation	Pyrosequencing of 16S rRNA gene	● Methanogens and syntrophic bacteria became less abundant ● *Acetanaerobacterium* and *Ruminococcus* became more abundant	Tian et al. (2015)
Batch	Thermophilic	Kitchen waste	● Initial inhibitory concentration of acetate was between 1.5 g L^{-1} –2.5 g L^{-1} ● Methanogenic activity was inhibited completely at VFA concentration of 5.8 g L^{-1} –6.9 g L^{-1}	PCR-DGGE	● Accumulation of acetic acid inhibited acetoclastic methanogens more than hydrogenotrophic methanogens	Xu et al. (2014)

Abbreviations: VFA, volatile fatty acids; *fthfs*, formyltetrahydrofolate synthetase-encoding gene; CSTR, continuous stirred tank reactor; N/A, not available; PCR-DGGE, polymerase chain reaction – denaturing gradient gel electrophoresis.

microbial communities. Nucleic acid–based molecular methods have revolutionized environmental biotechnology research by making it possible to study microbial communities without culturing (Kumaraswamy et al., 2014). DNA fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) have been employed widely to study AD systems. However, DNA fingerprinting and other early molecular methods suffer from limited coverage and depth. This limitation conflicts with the high diversity and importance of low abundance populations within the AD microbiome, making it difficult to accurately monitor the activity of rare, but functionally important microorganisms. Thus, sequencing-based approaches using pyrosequencing, Illumina sequencing, or other high-throughput platforms are now widely used to study AD systems. We can also couple high-throughput sequencing with isotope tracing (i.e., DNA- and RNA-stable isotope probing (SIP)) to link substrate uptake with specific microbial populations (Werner et al., 2014). Using advanced molecular tools, we now know that syntrophic acetate oxidation (SAO), a thermodynamically unfavorable reaction (De Vrieze and Verstraete, 2016), is possible by more diverse microbial populations than originally assumed (Lee et al., 2015; Treu et al., 2016; Werner et al., 2014). However, we continue to struggle with connecting community structure and function in AD systems (Carballa et al., 2015; De Vrieze and Verstraete, 2016) and have not yet unraveled the ‘black-box’ microbial ecology of AD (Nobu et al., 2015).

The AD research community has made considerable progress in understanding inhibitory impacts and mitigation strategies using advanced molecular methods. However, this work has not been critically reviewed to summarize recent progress and highlight gaps in our understanding of inhibition within the AD microbiome. The objective of this manuscript is to review comprehensively how molecular methods have improved our understanding of AD inhibition and highlight emerging molecular tools that could be used to prevent inhibition.

2. Inhibition of metabolic pathways in AD

2.1. Hydrolysis and fermentation

Hydrolysis, the first step in AD, is performed by hydrolytic fermentative bacteria that degrade complex polymers to oligomers and monomers using extracellular enzymes (e.g., cellulases, proteases, and lipases) (Keating, 2015). Complex insoluble polymers in many AD feedstocks can result in hydrolysis being rate limiting (Pavlostathis and Giraldo-Gomez, 1991). Hydrolytic bacteria in AD are found within five phyla: *Firmicutes*, *Bacteroidetes*, *Fibrobacter*, *Spirochaetes*, and *Thermotogae* (Azman et al., 2015). *Firmicutes* and *Bacteroidetes* are typically the most abundant taxa of hydrolytic bacteria in AD, although relative abundance of these taxa is often dictated by inoculum and reactor type, as reviewed by Azman et al. (2015). Following hydrolysis, fermentative bacteria degrade oligomers and monomers into intermediates such as VFAs and alcohols (acidogenesis).

Hydrolytic bacteria are inhibited by elevated levels of VFAs, LCFAs, hydrogen partial pressure, and humic acids (Azman et al., 2017; Azman et al., 2015; Cazier et al., 2015) (Tables 1–4). Inhibition occurs via activity loss, reversible reduction of hydrolases (e.g., when inhibitors bind to enzyme active sites or substrate-enzyme complexes), or irreversible impacts resulting from changes in enzyme chemical structure (Azman et al., 2015). The latter is challenging to mediate, requiring removal of the inhibitor from the system. Siegert and Banks (2005) found that VFA concentrations of 2 g L^{-1} resulted in 75% inhibition in cellulose hydrolysis. Similarly, another study reported inhibition of cellulose hydrolysis when VFAs exceeded 1.8 g L^{-1} (Romsaiyud et al., 2009). High hydrogen partial pressure was also found to be inhibitory to hydrolytic bacteria, reducing degradation of wheat straw with no accumulation of metabolites from acidogenic bacteria (Cazier et al., 2015). In another study, high humic acid concentrations resulted in a decrease of hydrolysis by 40% with this decline attributable to a

Table 2
Studies investigating ammonia inhibition.

Reactor	Temp.	Feed	Performance	Microbial community analysis tool	Effect on microbial community	Reference
CSTR	Mesophilic	Dewatered sludge	● VFA reduction changed from 32% to 21% and biogas decreased from 12 L d^{-1} to 10 L d^{-1} when ammonia concentrations reached 5000–6000 mg N L^{-1}	Pyrosequencing of 16S rRNA gene and qPCR targeting *Methanosarcinaceae*	● *Methanosarcina* dominated archaeal population (resistant to ammonia stress)	Dai et al. (2016)
UASB	Mesophilic	Basal anaerobic (BAN) medium	● 5 g NH_4-N L^{-1} led to 25% lower methane yield	FISH and confocal laser	● Bioaugmentation of SAO co-culture was not possible because methanogens used in the co-culture (*Methanoculleus* spp.) had slow growth rate	Fotidis et al. (2013)
CSTR	Thermophilic	Cattle manure	● Methane production decreased by 37.7% after addition of cattle manure and accumulation of ammonia	Ion Torrent PGM sequencing targeting 16S rRNA gene	● Inhibition of methanogens except *Methanoculleus*	De Francisci et al. (2015)
Pilot-scale CSTR	Mesophilic	Kitchen waste	● TAN concentration of 4000 mg L^{-1} led to severe inhibition, acclimated reactor performed well at TAN 4293 mg L^{-1}	MiSeq targeting 16S rRNA gene	● Ammonia stress led to increase in relative abundance of *Firmicutes* and hydrogenotrophic methanogens, and decrease of acetolactic methanogens	Gao et al. (2015)
Full-scale	Thermophilic	Food waste	● FAN at 367 mg L^{-1} did not inhibit performance, but altered microbial community dynamics	Metaproteomics and metagenomics	● Acetoclastic methanogens showed low abundance yet some metabolic activity ● Dominate acetate removal was through SAOB ● Discovered novel uncultured SAOB that can also degrade LCFA	Hagen et al. (2017)
Batch	Thermophilic	Sodium acetate	● Reduced initial methane production in the uninhibited control reactors early in the experiment, however, methane production increased in the high ammonia reactors later in the experiment and matched uninhibited control reactors by day 25.	Isotope labeled substrate assays and DNA-SIP with analysis of 16S rRNA gene pyrotags	● *Methanosarcina* performed acetoclastic methanogenesis at free ammonia nitrogen concentrations of up to 916 mg L^{-1}	Hao et al. (2015)
CSTR	Mesophilic	Sodium propionate and nutrient medium	● TAN at 3.0 g L^{-1} inhibited propionate degradation and methane recovery rate dropped from dropped from 82.91% to 28.09%	FISH	● Low abundance of methanogens under ammonia stress	Li et al. (2017b)
CSTR	Mesophilic	Thin stillage	● Performance threshold for ammonia concentration was 1 g NH_3 L^{-1}	qPCR targeting methanogenic populations, SAOB and *fthfs* OTUs, T-RFLP, and cloning	● *Methanoculleus* increased and acetogenic community decreased at high ammonia	Moestedt et al. (2016)
CSTR	Thermophilic	Chicken manure	● Biogas production almost ceased at high concentration of TAN (8000 mg L^{-1}) and VFA (25,000 mg N L^{-1})	16S rRNA gene cloning and sequencing	● Hydrogenotrophic methanogens dominated during inhibition phase while acetoclastic methanogens were inhibited	Niu et al. (2013)
CSTR	Mesophilic	Chicken manure and maize silage	● TAN at 7 g N L^{-1} critical threshold for performance	Isotope tracer	● Methanogens completely inhibited at TAN > 9 g N L^{-1}	Sun et al. (2016)
CSTR	Mesophilic	Thermally hydrolyzed waste activated sludge	● Methane production improved by 54% when ammonia concentration decreased from 630 to 92 mg L^{-1}	qPCR targeting methanogens	● Six-fold increase of *Methanosarcinaceae* and doubling of bacterial density improved VFA, protein, and carbohydrate removal	Tao et al. (2017)
Sequencing batch reactors	Mesophilic	Swine waste	● TAN at 4.4 g N L^{-1} and FAN at 0.08 g N L^{-1} decreased biogas production rate and VFA accumulation occurred	Isotope tracer, shotgun sequencing, cloning-sanger sequencing, DNA-SIP, and FISH-NanoSIMS	● Community shift from acetoclastic methanogenesis to SAO ● Decrease in community evenness associated with ammonia-induced stress	Werner et al. (2014)
Intermittent CSTR	Mesophilic	Food waste leachate	● FAN at 700 mg N L^{-1} resulted in significant inhibition	Pyrosequencing of 16S rRNA gene	● FAN inhibited *Methanosarcina* and *Methanosaeta*	Yun et al. (2016)

Abbreviations: UASB, upflow anaerobic sludge blanket; FAN, free ammonia nitrogen; FISH, fluorescent in situ hybridization; VFA, volatile fatty acids; *fthfs*, formyltetrahydrofolate synthetase-encoding gene.

Table 3
Studies investigating long chain fatty acid (LCFA) inhibition.

Reactor	Temp.	Feed	Performance	Microbial community analysis tool	Effect on microbial community	Reference
Batch	Thermophilic	FOG and food waste	● > 50% FOG volatile solid loading addition resulted in more than 90% decline in biogas production	Illumina MiSeq targeting 16S rRNA	● Increased relative activity of syntrophs (mostly *Syntrophomonas*), prevented LCFA accumulation in 30% FOG (v/v) addition ● > 50% FOG volatile solid loading addition was inhibitory to syntrophs and methanogens	Amha et al. (2017)
CSTR	Thermophilic	Oleate	● Up to 2 g oleate L^{-1} day^{-1} did not show inhibition and resulted in methane increase	PCR-DGGE	● *Firmicutes* increased abundance ● Decrease in bacterial diversity ● *Methanosarcina* and *Methanococcus* were the dominant methanogens	Baserba et al. (2012)
CSTR	Thermophilic	Cattle manure and sodium oleate	● In non-acclimated reactor LCFA addition of 3 g L^{-1} resulted in 95% reduction in methane yield ● Inhibition was reversible	Metagenomics	● *Syntrophomonas* and *Methanosarcina* increased in relative abundance ● Microbes that responded positively to LCFA pulse could encode proteins related to "chemotaxis" and "flagellar assembly"	Kougias et al. (2016)
Batch	Mesophilic	Lipid-extracted algal biomass	● In the high lipid concentration digester, inoculum/substrate ratios of < 1 resulted in biogas production decline	Illumina MiSeq targeting 16S rRNA gene	● Bacterial community was affected more than methanogens at high LCFA ● Hydrolytic bacteria and acetoclastic methanogens dominated ● Syntrophic acetogens were sensitive to high LCFA	Ma et al. (2015)
Various	Mesophilic	Skim milk oleate	● Long term acclimation to LCFA was found necessary to prevent inhibition	n/a	● Exposure to LCFA > 100 days resulted in acclimated microbial community and limited inhibition ● Bioaugmentation of *Syntrophomonas zehnderi* and *Methanobacterium formicicum* showed no performance improvement	Silva et al. (2014)
Batch	Mesophilic	Oleate, stearate, and palmitate	● Methanogenic activity decreased by 50% with 0.3, 0.4 and 1 mM oleate, stearate, and palmitate with *M. hungatei* cultures, whereas 50% reduction in methanogenic activity was seen at 1 mM oleate and > 4 m stearate or palmitate for *M. formicicum* culture	Live/Dead BacLight bacterial viability kit, cloning and sequencing, DGGE	● *Methanobacterium formicicum* was more resilient than *Methanospirillum hungatei* for both saturated and unsaturated LCFA ● *Methanosarcina mazei* and *Methanosaeta concilii* were inhibited in oleate culture and acetate accumulated	Sousa et al. (2013)
CSTR	Thermophilic	Cattle manure and sodium oleate	N/A	Metatranscriptomics	● *Syntrophomonas* dominated at high LCFA concentrations ● Protective mechanisms was suggested as upregulation of genes involved in peptidoglycan and lipopolysaccharides biosynthesis	Treu et al. (2016)
CSTR	Mesophilic	Manure and oleate	● Effluent acetate reached 3000 mg L^{-1} in continuous fed reactor	Illumina MiSeq targeting 16S rRNA gene	● The relative abundance of *Syntrophomonas* increased to ~15% ● *Methanosaeta* and *Methanospirillum* were the dominant methanogens	Ziels et al. (2016)
CSTR	Mesophilic	Oleic acid	● After acclimation (on day 204), the continuously fed reactor was inhibited at oleate concentration of 875 mg L^{-1}, whereas the pulse-fed reactor was not inhibited at 1800 mg L^{-1} oleate concentration	Illumina MiSeq targeting 16S rRNA gene and qPCR targeting *Syntrophomonas*, total bacteria, and total archaea	● LCFA feeding frequency and OLR impacted microbial community composition and biokinetics ● Higher relative and absolute 16S rRNA gene concentration of *Syntrophomonas* and *Methanosaeta* in pulse feed digester than continuously fed digester	Ziels et al. (2017)

Abbreviations: PCR-DGGE, polymerase chain reaction – denaturing gradient gel electrophoresis; qPCR, quantitative polymerase chain reaction.

Table 4
Miscellaneous studies investigating inhibition.

Inhibitors	Reactor	Temp.	Feed	Performance	Microbial community analysis tool	Effect on microbial community	Reference
Humic acid	Batch	Mesophilic	Cellulose and xylan mixture	• Biogas production decreased approx. 33% at inhibited conditions	Illumina HiSeq targeting 16S rRNA gene	• Inhibited the hydrolysis efficiency of the digestion by 40%	Azman et al. (2017)
H_2	Batch	Mesophilic	Wheat straw	• Substrate degradation decreased without accumulation of metabolites from acidogenic bacteria	Illumina MiSeq targeting 16S rRNA gene	• Hydrolytic activity was impacted more than acidogenic bacteria	Cazier et al. (2015)
Humic acid	Batch	Mesophilic	HAL and FAL substances from maize and cow manure	• Tributyrin hydrolysis was inhibited by HAL from 0.5 to 5.0 g L^{-1}	T-RFLP fingerprinting targeting 16S rRNA gene	• Cellulose hydrolysis was inhibited by 0.5 to 5.0 g L^{-1} of HAL and FAL	Fernandes et al. (2015)
Methyl Fluoride	Batch	N/A	Sodium acetate	• Reduced biogas formation observed with increasing concentrations of CH_3F in the headspace.	Stable isotope analysis of biogas	• Acetoclastic methanogenesis was progressively inhibited in the presence of CH_3F at low and middle concentrations • At the highest concentration of CH_3F (10% in the headspace), both acetoclastic and hydrogenotrophic methanogenesis were inhibited	Hao et al. (2011)
Starvation	CSTR	Thermophilic	Cattle manure	• VFAs, H_2S, and ammonia accumulated during starvation phase	Illumina MiSeq targeting 16S rRNA gene	• SAOB and hydrogenotrophic methanogens increased after inhibition phase	de Jonge et al. (2017)
Dissolved lignin	CSTR	Mesophilic and thermophilic	Lignin-rich indigenous macrophyte species	• Performance decreased by approx. 40% in pretreated substrate during inhibition	Cloning and sequencing of 16S rRNA gene	• Hydrogenotrophic methanogenic pathway was a limiting step for alkaline treated reactors, where increase in partial pressure of H_2 caused accumulation of VFAs	Koyama et al. (2017)
OLR	CSTR	Mesophilic	Food waste	• Organic load increase to 6 g VS L^{-1} d^{-1} led to 50% reduction in methane production	pyrosequencing of 16S rRNA gene	• Acid producing bacteria and syntrophic fatty acid oxidizers increased in relative abundance at high OLR • Acetoclastic methanogens dominated	Li et al. (2015)
OLR	CSTR	Mesophilic	Food waste	• OLR > 6 g VS L^{-1} d^{-1} resulted in increase of FAN (114 mg L^{-1}) and VFA (9443 mg L^{-1} at day 90) • Propionate increased by 20-fold	pyrosequencing of 16S rRNA gene	• Acidogenic bacteria showed functional redundancy and adopted to high OLR • Acetogens such as the genera *Syntrophomonas* and *Treponema* increased with high VFA • Hydrogenotrophic methanogens were inhibited	Treu et al. (2016)
Total solids	CSTR	Mesophilic	Sewage sludge	• TS increase from 10% to 15% resulted in biogas production decrease from 383 mL g VS^{-1} to 316 mL g VS^{-1}	Illumina MiSeq targeting 16S rRNA gene	• Acetoclastic methanogens decreased along with the increased TS • Hydrogenotrophic methanogens increased • Acidogenic and acetogenic bacteria of phylum *Firmicutes* decreased but phylum *Bacteroidetes* increased in relative abundance	Liu et al. (2016)
OLR	Semi-CSTR	Mesophilic	*Spirulina*	• Increasing the OLR from 0.5 to 1 g *Spirulina* L^{-1} d^{-1} led to inhibition	Metatranscriptomics and metagenomics	• Hydrolysis was mainly performed by *Bacteroides* while metagenomic activity was dominated by *Methanocalculus*	Nolla-Ardèvol et al. (2015)
2-BES	Mesocosm	Mesophilic	cow dung and sludge from WWTP	• 0.5 mmol L^{-1} BES and 10 mmol $LBES^{-1}$ led to 89% and 100% methane production reduction	RT-qPCR targeting mcrA	• Methanogenic activity decreased with the exposure to the inhibitors • Acetoclastic methanogens were more impacted than hydrogenotrophic methanogens	Webster et al. (2016)

Abbreviations: OLR, organic loading rate; T-RFLP, terminal restriction fragment length polymorphism; FAN, free ammonia nitrogen; 2-BES, 2-bromoethanesulfonate; VFA, volatile fatty acids; TS, total solids; *fthfs*, formyltetrahydrofolate synthetase-encoding gene; WWTP, wastewater treatment plant.

decrease in relative abundance of hydrolytic/fermentative bacterial populations including *Clostridiales*, *Bacteroidales,* and *Anaerolineales* (Azman et al., 2017). Inhibition due to LCFA has also been reported, with inhibition of hydrolytic bacteria occurring at 2.6–9.4 kg COD m^{-3} and acidogenic bacteria at a similar concentration range, 2.1–7.9 kg COD m^{-3} (Ma et al., 2015).

2.2. Syntrophy and methanogenesis

Syntrophic bacteria in AD convert fatty acids produced by acidogenic bacteria into acetate, hydrogen, and carbon dioxide. A total of 23 different genera have been identified to date with the ability to function as syntrophic bacteria, with most syntrophic genera found within *Firmicutes* (Schuchmann and Müller, 2014). Of these syntrophs, two families, *Syntrophomonadaceae* and *Syntrophaceae*, and 14 species within these families are able to degrade LCFAs (Baserba et al., 2012; Sousa et al., 2009). Thermodynamically, LCFA fermentation is both endothermic and nonspontaneous (Chen et al., 2014a). Therefore, LCFA degradation to acetate and hydrogen is made possible through β-oxidation and syntrophy with hydrogenotrophic or acetoclastic methanogens (Chen et al., 2014a; Treu et al., 2016; Ziels et al., 2017).

Methanogens are commonly categorized as hydrogenotrophic or acetoclastic based on their electron donor. Many hydrogenotrophic methanogens can metabolize C1 compounds such as formate and methanol in addition to hydrogen (Demirel and Scherer, 2008). *Methanosaeta*, obligate acetoclastic methanogens, have been shown to dominate in mesophilic AD (Guo et al., 2015a) and outcompete *Methanosarcina* at low acetate concentration due to their higher substrate affinity (Conklin et al., 2006). *Methanosarcina*, unlike *Methanosaeta*, are mixotrophic methanogens that can metabolize acetate, hydrogen, and C1 compounds (Mladenovska and Ahring, 1997). Some studies have suggested that hydrogenotrophic methanogens are dominant at thermophilic temperatures (Pap et al., 2015) and during inhibition due to high VFA and ammonia (de Jonge et al., 2017).

Common inhibitors of syntrophs and methanogens are VFAs, ammonia, and LCFAs (Chen et al., 2008) (Tables 1–3). Relative to methanogens, syntrophic bacteria better tolerate high VFA and ammonia (Treu et al., 2016). In fact, high VFA could initially promote the growth of syntrophic bacteria (Li et al., 2015; Treu et al., 2016). However, inhibition of hydrogenotrophic methanogens, their syntrophic partners, at elevated VFA concentrations destabilizes syntrophy, eventually leading to increased hydrogen partial pressure and a decrease in thermodynamic favorability of the reaction (Li et al., 2015). A study that investigated the effect of VFA accumulation due to high organic load in a batch reactor treating kitchen waste found that VFAs at 5.8–6.9 g L^{-1} were completely inhibitory to methanogens (Xu et al., 2014). In contrast, high VFAs were shown to be non-inhibitory to methanogens in a full-scale reactor treating cow manure and food waste, even when propionate concentrations reached 8.7 g L^{-1} (Franke-Whittle et al., 2014). Regarding ammonia inhibition, studies have reported that acetoclastic methanogens are inhibited more severely than hydrogenotrophic methanogens (Hagen et al., 2017; Niu et al., 2013; Sun et al., 2016; Werner et al., 2014). For example, in one study methanogenesis was completely inhibited when total ammonia nitrogen (TAN) exceeded 9 g N L^{-1} with the critical threshold for performance decline reported to be 7 g N L^{-1} (Sun et al., 2016). Methanogens also compete with other populations for substrates including sulfate reducing bacteria (SRB) that also produce potentially toxic sulfides. Hydrogen sulfide has been shown to diffuse through cell membranes and form disulfide cross-links between polypeptide chains, thereby denaturing proteins and affecting cellular function (Chen et al., 2014a; Tursman and Cork, 1989).

Syntrophic bacteria have been found to be particularly sensitive to LCFA, with syntrophic acetogens decreasing in abundance at high LCFA concentrations (Ma et al., 2015). Ma et al. (2015) proposed that inhibition resulted from attachment of LCFA on cell surfaces limiting mass transfer and substrate access. Although fatty acid-based inhibition is not well understood, researchers have identified several likely mechanisms of inhibition: disruption of the electron transport chain and oxidative phosphorylation, interference with cellular energy production, direct lysis of bacterial cells, and decreased cell permeability (Desbois and Smith, 2010; Pereira et al., 2005). However, some studies have suggested that LCFA inhibition is reversible (Kougias et al., 2016; Pereira et al., 2005; Ziels et al., 2016). For example, a study involving cattle manure digestion with the addition of sodium oleate to simulate high LCFAs reported that the perturbation observed was reversible (Kougias et al., 2016). Inhibition was also minimized when inoculating with sludge previously acclimated to high LCFAs (Kougias et al., 2016), suggesting microbial community adaptation can lead to resilience. Notably, resilience was found to be an important factor in maintaining a syntrophic population exposed to disturbances in a full-scale reactor, where syntrophs rebounded following stress conditions (Werner et al., 2011). Another study also suggested reversibility of LCFA inhibition due to an increase in relative abundance of syntrophic β-oxidizing bacteria, primarily *Syntrophomonas* (Ziels et al., 2016). Further, the same study reported that the abundance and composition of methanogens was unaffected by addition of 100–1570 mg oleic acid g VS^{-1}, where 70% of the methanogens were hydrogenotrophic (*Methanomicrobiales*) and 30% were acetoclastic (*Methanosaeta*). Similarly, another study reported that increase of LCFA affected hydrolytic bacteria more so than methanogens (Ma et al., 2015). LCFA inhibition is further complicated by observations of varying inhibition due to type of LCFA, saturated versus unsaturated LCFAs, and methanogen taxonomy. Sousa et al. (2013) investigated the impact of high concentrations of oleate (unsaturated LCFA) and palmitate (saturated LCFA) on pure cultures of acetoclastic methanogens and hydrogenotrophic methanogens, finding that saturated LCFA had greater inhibitory effects than unsaturated LCFA on methanogens and that the inhibition mechanism was through damaged membrane integrity. Further, *Methanobacterium formicicum* (hydrogenotrophic) was more resilient than *Methanospirillum hungatei* (hydrogenotrophic) for both saturated and unsaturated LCFA whereas *Methanosarcina mazei* (acetoclastic) and *Methanosaeta concilii* (acetoclastic) were completely inhibited by oleate.

Inhibition of acetoclastic methanogens can also drive increases in syntrophic acetate oxidizing bacteria (SAOB), which oxidize acetate into hydrogen and carbon dioxide, that in turn can increase hydrogenotrophic methanogenic activity (Carballa et al., 2015; Gao et al., 2015). SAOB remain poorly studied, with only a few cultured populations to date: *Syntrophaceticus* spp., *Thermacetogenium phaeum*, *Thermotoga lettingae*, *Tepidanaerobacter acetatoxydans*, and *Clostridium ultunense* (Müller et al., 2013). Some studies suggest that SAO could be conducted by more diverse populations than previously thought (Lee et al., 2015; Werner et al., 2014). For example, a recent study identified cluster II *Spirochaetes* and members of *Clostridia* as potential SAOB using ^{13}C-labeled acetate (Mosbæk et al., 2016). Ammonia induced perturbation has also been shown to promote the SAO pathway, with a shift from acetoclastic to hydrogenotrophic methanogenesis (Werner et al., 2014).

The impact of inhibitors may be heavily dependent on microbial community acclimation, with long-term exposure often leading to stable community function in the presence of inhibitors (Silva et al., 2014; Silvestre et al., 2011). Therefore, it is unsurprising that a wide range of inhibitor concentrations have been reported to date (Tables 1–4). This variability likely stems from differences in inoculum (Baserba et al., 2012) or prior exposure to inhibitors. Differences in microbial community structure make it particularly challenging to compare inhibition in AD. The majority of studies to date have only applied DNA-based methods to study microbial community structure during AD inhibition (de Jonge et al., 2017; Li et al., 2015; Ma et al., 2015; Treu et al., 2016; Ziels et al., 2016). However, DNA-based methods are relatively insensitive, particularly in anaerobic communities with low biomass yields. Inhibitory conditions negatively impact

DNA replication rates and DNA may persist in the environment after a cell ceases activity. De Vrieze et al. (2016) demonstrated that presence does not always correlate with activity by studying community response to salt perturbation. The authors also used RNA-based sequencing and found that it was a more sensitive tool than DNA-based sequencing to quantify microbial community response to high salt concentrations. For example, *Methanosaeta* remained the most abundant methanogen based on DNA-sequencing, but was strongly inhibited by high salt concentration according to RNA-based sequencing, corroborating performance observations. Similar observations have been reported during high FOG addition (Amha et al., 2017), providing further evidence supporting the need for RNA-based methods when evaluating AD inhibition. Recent developments in molecular methods have resulted in a greater understanding of inhibition in AD, as discussed in subsequent sections.

3. Targeted nucleic acid biomarkers

3.1. Universal target: 16S rRNA gene and metadata analysis

The 16S rRNA gene is the most widely used biomarker and provides detailed phylogenetic information about Bacteria and Archaea from mixed microbial communities. Several limitations of this approach should be noted. Because the number of copies of the 16S rRNA gene per genome can vary from 1 to 15 and is often not known, differences in relative abundances can often misrepresent the true abundances of populations (Klappenbach et al., 2000). Even within the same organism, the number of copies of rRNA genes will differ at different stages of development and in the metabolic state at the point of sampling, i.e. dormant, active or growing (Blazewicz et al., 2013; Sukenik et al., 2012). Relative abundance information can also be misleading when the overall size of the community is changing (Props et al., 2016), though quantitative sequencing methods are beginning to be used (Smets et al., 2016). Despite these limitations, 16S rRNA gene sequencing remains one of the most convenient and widely used methods to characterize a microbial community.

We aimed to draw on data from existing 16S rRNA gene sequence datasets to investigate how microbial communities vary across AD over a range of fatty acid concentrations. We collected 16S rRNA gene sequence data and metadata available from four different publicly available studies that included a total of 99 samples from bench- and full-scale ADs operated at mesophilic and thermophilic temperatures (Amha et al., 2017; Liu et al., 2016; Ma et al., 2015; Treu et al., 2016). These studies were selected based on sequence data and metadata availability and their comparison of LCFA and VFA inhibition.

Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010) was used for the pre-processing and downstream analyses after the samples were pooled together. Closed-reference operational taxonomic unit (OTU) calling was performed against Greengenes (DeSantis et al., 2006) (version 13.8) as a reference database. OTUs were assigned using QIIME implementation of the UCLUST_ref (Edgar, 2010) algorithm for clustering, with a threshold of 97% similarity. Beta-diversity was calculated after adaptive rarefaction (Henschel et al., 2015) using a Bray Curtis matrix and hierarchical clustering. Samples were pooled based on categorical levels of LCFA and VFA.

In the majority of the compiled samples, *Proteobacteria* (particularly *Gammaproteobacteria* and *Deltaproteobacteria*), methanogens (*Methanobacteria* and *Methanomicrobia*), and *Firmicutes* (*Clostridia*) were present at high relative abundance irrespective of study and LCFA or VFA concentration (i.e., level of inhibition). The LCFA analysis showed that *Synergistia*, *Clostridia*, and *Bacilli* were less impacted by high LCFA concentrations relative to other groups (Fig. 1A). In fact, the relative abundance of *Bacilli* increased with increasing LCFA concentrations. *Thermotogae* were highly impacted by high LCFA concentrations and showed negligible relative abundance in most of the samples with high LCFA concentrations. In contrast, high VFA concentrations impacted *Synergistia*, *Clostridia*, and *Bacilli* in most of the samples (Fig. 1B). Although there were a few samples that showed high relative abundance of *Clostridia* and *Bacilli* under high VFA concentration, these groups were absent in most samples at high VFA concentration. Similar to the LCFA analysis, *Thermotogae* decreased with increasing VFA concentration, whereas, *Methanomicrobia* increased in relative abundance with increasing VFA concentration in the majority of the samples. Despite some visual trends in key populations across LCFA and VFA concentrations, we did not find any statistical correlation between changes in taxa relative abundance and LCFA or VFA concentration.

To address biases that arise when comparing data from different studies, a core microbiome approach (Huse et al., 2012) was utilized. Our analysis was limited by sample size given that sequence data and/or metadata is not always available in public datasets or made available upon request. It is also important to note that sequencing technology and the region of the 16S rRNA gene targeted can vary between studies and introduce biases. The 16S rRNA gene is approximately 1500 bases long but most next generation sequencing platforms only sequence a small section (e.g., 250 bp for Illumina MiSeq). Therefore, primers target variable regions of the 16S rRNA gene (V1-V9). In our analysis, the included sequence datasets targeted different variable regions within V2-V5 of the 16S rRNA gene. The core microbiome was computed separately for LCFA and VFA samples using QIIME to identify the OTUs present in at least 60% of the samples.

Across the range of LCFA concentrations, four classes were identified in the core microbiome: *Methanobacteria, Clostridia, Bacteroidia,* and *Synergistia* (Fig. 2A). The relative abundance of *Bacteroidia* and *Clostridia* increased with increasing LCFA concentration, whereas *Methanobacteria* decreased. This is consistent with the expectation that microorganisms with fatty acid metabolisms would dominate at higher fatty acid concentrations. *Synergistia* were the most resistant to increasing LCFA and remained least affected relative to other populations. The core microbiome for VFA samples included *Methanomicrobia, Methanobacteria, Clostridia, Bacteroidia,* and *Synergistia.* Similar to the LCFA analysis, *Bacteroidia* and *Clostridia* increased in relative abundance at higher VFA concentrations while methanogen relative abundance (*Methanobacteria* and *Methanomicrobia*) decreased. *Bacteroidia* were the only population from the core microbiome still present at inhibitory VFA concentrations (Fig. 2B). These results suggest that key phylogenetic groups involved in AD respond similarly to fatty acid inhibition across different digesters and operational conditions.

Our meta-analysis effort was significantly confounded by two factors: data heterogeneity and limited data availability. Data heterogeneity largely stems from DNA/RNA extraction protocol, PCR primer selection, sequencing platform, sequencing depth, etc. Efforts should be made to: (i) standardize data sharing by depositing raw data in publicly available databases with supporting information on sample demultiplexing, primers used, etc.; and (ii) measure and provide metadata describing environmental conditions such as pH, temperature, LCFA, VFA, ammonia, etc. in standard quantitative units. Better availability of high-quality sequencing data alongside metadata would help the AD community construct a theoretical framework describing microbial community dynamics in the presence of inhibitors.

3.2. Functional genes

Another drawback of the 16S rRNA gene is that it cannot be tied to a specific metabolic function, and therefore, it can be difficult to infer the contribution of a given population to changes in the biochemical environment (Blazewicz et al., 2013). However, many functional genes related to AD have been characterized and can be used to target specific populations. For hydrogen producing fermentative bacteria, the use of genes encoding the large subunit of Fe–Fe-hydrogenase (*hydA*) have been used as biomarkers (Xing et al., 2008; Ziganshin et al., 2016). To study populations that degrade aromatic compounds, which is important when considering AD of petrochemical industry waste and

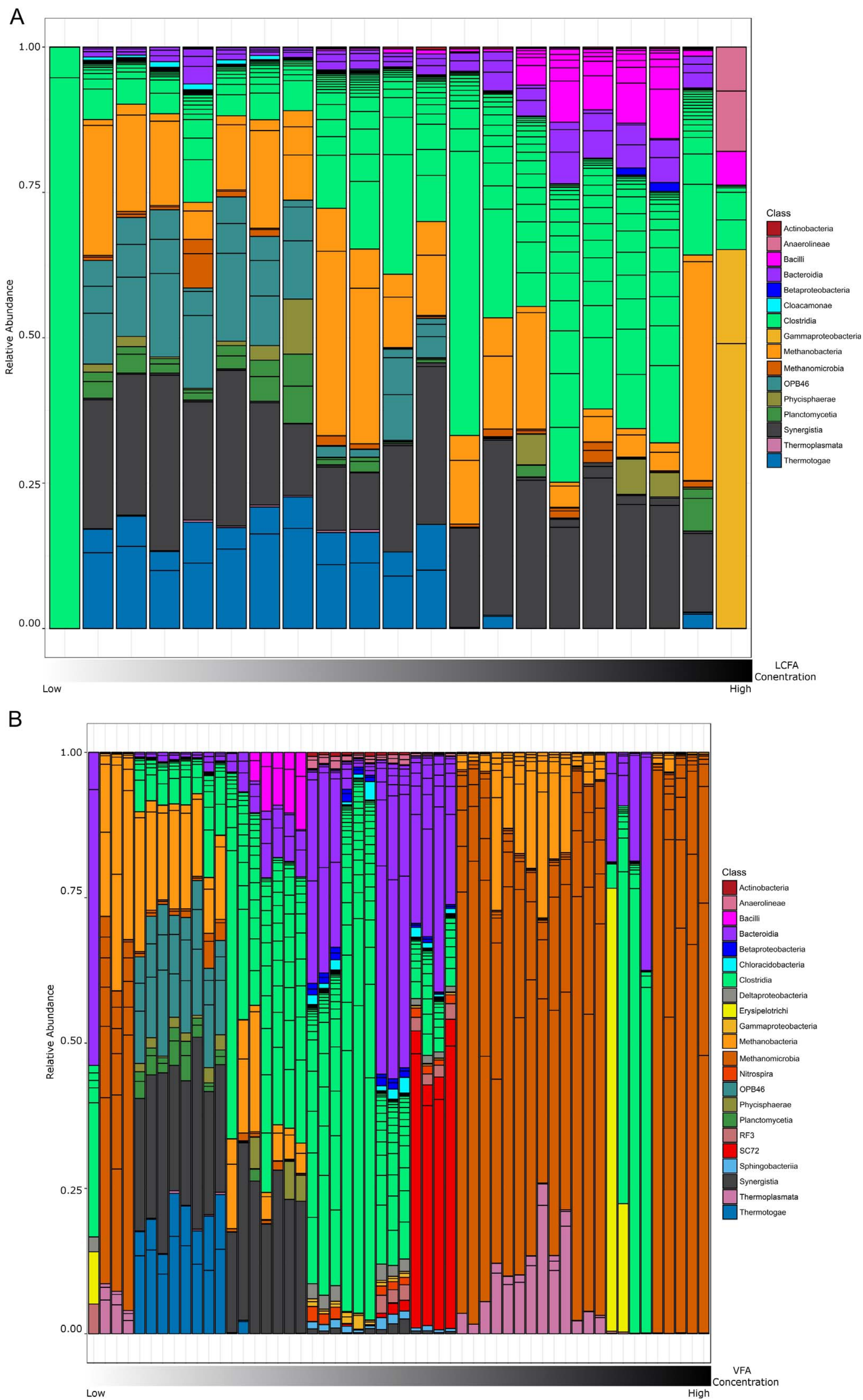

Fig. 1. (A) Microbial community structure by class over increasing LCFA concentration (left to right). The y-axis represents relative abundance of OTUs ≥ 0.1% of the community. Stacked bars within each class (same color) represent orders. (B) Microbial community structure by class over increasing VFA concentration (left to right). The y-axis represents relative abundance of OTUs that are ≥ 0.1% of the community. Stacked bars within each class (same color) represent orders. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

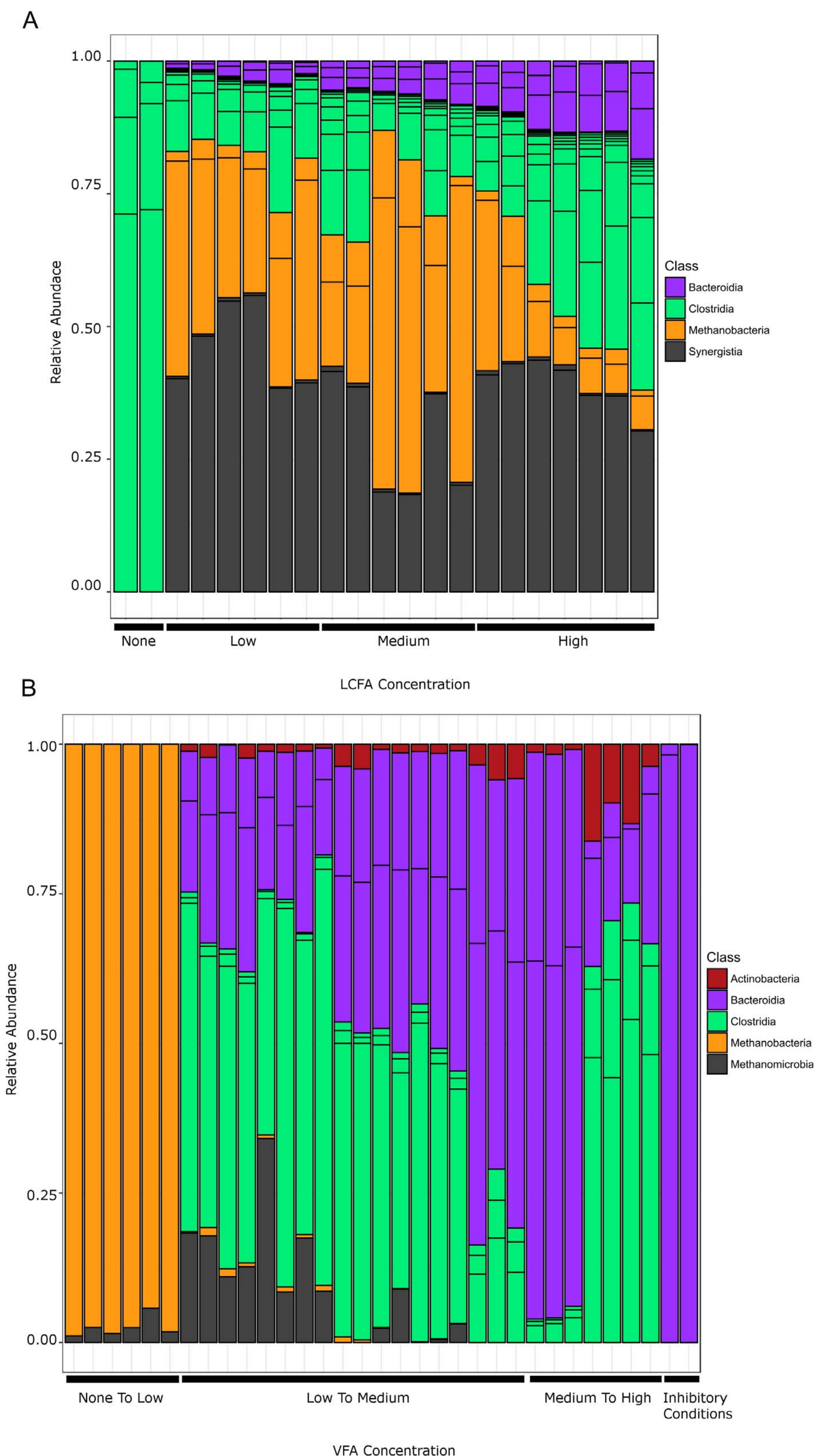

Fig. 2. (A) Core microbiome by class over increasing LCFA concentration (left to right). The y-axis represents relative abundance of populations within the core microbiome and is therefore independent of populations outside of the core microbiome. Stacked bars within each class (same color) represent orders. (B) Core microbiome by class over increasing VFA concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phenol containing substrates, functional genes for the *benzoylcoenzyme A* (*benzoyl-CoA*) degradation pathway have been designed as biomarkers (Levén et al., 2012).

Although syntrophic bacteria have diverse metabolisms, the assimilation of CO_2 into biomass and conservation of energy using the acetyl-CoA pathway has made it possible to use the formyltetrahydrofolate synthetase-encoding gene (*fthfs*), a key enzyme for the acetyl-CoA pathway, also known as the Wood-Ljungdahl pathway as a biomarker (Müller et al., 2013). SAOB oxidize acetate to carbon dioxide and hydrogen using the reverse Wood-Ljungdahl pathway, and various studies

have used the *fthfs* gene as a biomarker to identify and quantify SAOB in AD (Mosbæk et al., 2016; Müller et al., 2013) although it has also been noted that some non-acetogenic bacteria may also have this gene (Lovell and Leaphart, 2005; Mosbæk et al., 2016).

Methanogens are often targeted in AD as indicators of performance because their presence and activity has been shown to correlate with biogas production measurements (Morris et al., 2014; Webster et al., 2016). The most common functional gene used to study methanogens encodes for the α-subunit of the methyl coenzyme-M reductase (*mcrA* gene), and has been suggested as the most important biomarker in AD systems (De Vrieze and Verstraete, 2016). Significant positive correlations between methane production rate and *mcrA* gene copy numbers (Morris et al., 2014) and transcripts (Webster et al., 2016) have been shown. An alternative method to target *mcrA* without having to extract mRNA, is measuring the coenzyme F430, a coenzyme of *mcrA* gene, using liquid chromatography/mass spectrometry. This method can detect methanogens in the range of 600–10,000 cells (Kaneko et al., 2014). Although this method has been used to quantify methanogens in environmental samples (Kaneko et al., 2014; Takano et al., 2013), more studies should apply the method in AD systems and explore techniques to reduce the detection limit.

4. Omics studies

4.1. Metagenomics

Metagenomics, "shotgun" sequencing of environmental DNA, is an approach to characterizing structure and metabolic potential that can provide greater information than amplicon gene sequencing approaches discussed in the preceding section. New sequencing platforms have steeply decreased costs, enabling sufficient sequencing depth for metagenomics on environmental communities (Vanwonterghem et al., 2014). Notably, metagenomics does not rely on PCR amplification, thus eliminating concerns regarding amplification efficiency or primer biases. Further, unlike targeting the 16S rRNA gene, sequence data from metagenomics can be used to infer functional potential of a microbial community without relying on taxonomy-based physiological characteristics (Shah et al., 2010; Shakya et al., 2013).

Recent studies have applied metagenomics to study the microbial community response to inhibitors such as ammonia (Gao et al., 2015; Li et al., 2017a; Werner et al., 2014), LCFA (Beale et al., 2016; Kougias et al., 2016), temperature (Beale et al., 2016; Pap et al., 2015), and VFA (Mosbæk et al., 2016). A study that used [U-^{13}C] labelled acetate and metagenomics to evaluate VFA inhibition observed that acetate was consumed by *Methanosarcina*, *Methanoculleus*, and five subspecies of *Clostridia* that contained the *ftfhs* gene (Mosbæk et al., 2016). This indicated that the identified species of *Clostridia* were potential SAOB, as the *ftfhs* gene is a key enzyme for reductive acetogenesis. Metagenomics has also been used to characterize temperature-based competition between acetoclastic and hydrogenotrophic methanogens (Pap et al., 2015). At mesophilic temperature, acetoclastic methanogens dominated the archaeal community. However, a gradual increase to thermophilic temperature enriched hydrogenotrophic methanogens alongside an increased abundance of hydrogen producing Fe-hydrogenases associated with syntrophic and fermentative bacteria. Metagenomics has similarly been used to evaluate methanogenic pathways in response to elevated ammonia, with a shift from acetoclastic to hydrogenotrophic methanogenesis and SAO reported (Gao et al., 2015; Li et al., 2017a; Werner et al., 2014). Beyond revealing the shifts in microbial community composition and metabolic potential, metagenomics has also been effective at elucidating communities that would have remained uncharacterized without using non-targeted approaches (Guermazi et al., 2008; Kougias et al., 2017).

More recently, Pacific Biosciences (PacBio) has developed the single-molecule real time (SMRT) sequencing platform that is capable of long read lengths compared to other second generation sequencing platforms. According to a recent review, SMRT sequencing is capable of producing sequence reads averaging 10 kb, and as high as 60 kb (Rhoads and Au, 2015). Combining long read SMRT sequence data with high-throughput and high-accuracy sequences from second generation platforms such as Illumina HiSeq can facilitate the construction of longer and more accurate metagenome assemblies (Frank et al., 2016). These hybrid assemblies have also been used to study low-abundance and difficult to sequence phylotypes in AD systems (Hagen et al., 2017), and this technique could allow researchers to study the role that these phylotypes play during inhibition.

A major limitation of metagenomics is that it characterizes communities based on phylogeny and functional potential, not function according to gene expression or translated proteins. It is important to note that AD systems rely on low abundance/rare populations (e.g., syntrophic bacteria), which can be challenging to accurately quantify using molecular approaches (Shah et al., 2010). A study comparing metagenomics and 16S rRNA gene sequencing found higher sensitivity and resolution using metagenomics, while 16S rRNA gene sequencing only captured broad shifts in microbial diversity over time (Poretsky et al., 2014). Another study used synthetic communities to compare metagenomics and 16S rRNA gene sequencing and reported that both Illumina and 454 metagenomics outperformed 16S rRNA gene sequencing (Shakya et al., 2013). Despite the drastic increase in nucleotide database coverage, the bottleneck for metagenomics remains genome assembly and gene prediction from assembled reads, particularly because environmental annotations are lacking (Cabezas et al., 2015). It is likely that future advancements in sequencing technology and algorithms for analysis will aid genome assembly from complex communities (Vanwonterghem et al., 2014).

4.2. Metatranscriptomics

Metatranscriptomics is an RNA-based molecular method that uses "shotgun" sequencing of reverse transcribed environmental RNA to characterize functional activity of a microbial community (Cabezas et al., 2015). The first study that used metatranscriptomics in AD compared 16S rRNA sequences from the metatranscriptome, 16S rRNA gene sequences from the metagenome, and 16S rRNA gene sequences generated via amplicon sequencing in a full-scale AD (Zakrzewski et al., 2012). In general, the most abundant microbes retrieved using metagenomics and amplicon sequencing also contributed the majority of 16S rRNA sequences. However, Archaea represented 2.4% and 12.9% of 16S rRNA gene relative abundance using metagenomics and amplicon sequencing, respectively, but contributed 24% of the transcriptional activity. This indicates that archaeal populations may have high transcriptional activity, even when constituting a relatively small fraction of the community based on gene abundance. More effective mRNA enrichment methods are needed to better analyze functional activity, as only 2.6% of the metatranscriptome reads were mRNA, with more than 90% of the reads rRNA. For this reason, pre-treatment to remove rRNA prior to sequencing has been investigated as a more targeted approach to understand community function (He et al., 2010). Further, RNA extraction efficiencies have been found to have biases in quantitative analysis of metatranscriptomics data (Stark et al., 2014).

Few studies to date have used metatranscriptomics to evaluate AD during inhibition (Nolla-Ardèvol et al., 2015; Treu et al., 2016). Treu et al. (2016) investigated inhibition by simulating high LCFA concentrations with oleic acid. Similar to other studies using 16S rRNA gene sequencing (Ziels et al., 2017; Ziels et al., 2016), *Syntrophomonas* increased activity at high LCFA concentrations. However, metatranscriptomics enabled elucidation of two potential mechanisms of adaptation to high LCFA concentration: (1) upregulation of genes involved in peptidoglycan and lipopolysaccharides biosynthesis that possibly result in membrane modification and (2) transcriptional activation of the chemotaxis genes that enable responses to fatty acids gradient (Treu et al., 2016). Further application of metatranscriptomics is likely to

expand our understanding of the mechanisms of inhibition and potential options to avoid digester failure.

4.3. Metaproteomics

Post-translational regulation of proteins prevents the accurate prediction of all activities based on gene expression measured by metatranscriptomics, a drawback that is avoided by metaproteomics (Cabezas et al., 2015). In metaproteomics, expressed proteins are characterized using three main steps: protein extraction, followed by separation/fractionation, and subsequent detection with mass spectrometry (Vanwonterghem et al., 2014). Metaproteomics is particularly useful in identifying novel functional systems and obtaining direct functional insights (Lü et al., 2014; Siggins et al., 2012). Further, application of metaproteomics with metabolomics, which measures intermediate cellular products, can provide new information related to changes in microbial activity, system function, and mechanisms of adaptation to inhibitory conditions (Siggins et al., 2012).

Some methodological challenges to widespread application of metaproteomics remain, including the extraction of high-quality protein at sufficient amounts, interference of co-extracted compounds, and the need for metagenomics data (unless de novo peptide sequencing is conducted) (Siggins et al., 2012). One metaproteomic study on AD of cellulose identified more than 500 non-redundant protein functions (Lü et al., 2014). The only study to our knowledge that used metaproteomics under potentially inhibitory conditions (free ammonia of 367 mg NH_3-N L^{-1}), found that acetoclastic methanogens were present at low abundance and enzymes associated with *Methanosaeta thermophile* were detected (Hagen et al., 2017). However, the dominant mechanism of acetate removal was found to be through SAO by groups closely related to *Thermacetogenium phaeum*. In fact, this study discovered two novel uncultured bacteria with necessary genes for both SAO and β-oxidation of LCFA. Although a number of recent studies have applied metaproteomics to study the functional activity of AD with various substrates (Jing et al., 2017; Kohrs et al., 2014) more studies are needed to elucidate changes in protein expression during inhibition.

5. Mapping substrate utilization

Anaerobic metabolisms form a complex web of substrate utilization mediated by a broad consortium of microorganisms. Inhibition of a single organism or group of organisms can prevent an intermediate substrate from being formed, which in turn may hinder subsequent metabolisms and ultimately biogas production. It is therefore useful to develop tools that allow researchers to pair substrate utilization with specific organisms to better understand the chain of substrate utilization in AD. Studying substrate utilization patterns and community dynamics under stressed and inhibited conditions provides information that can be used to develop more robust and reliable AD systems.

Stable isotope and radio isotope labeled compounds are used to link specific substrates with degradation products. Lettinga et al. (1999) fed batch anaerobic reactors with ^{14}C labeled acetate, ^{14}C labeled bicarbonate, and unlabeled propionate and analyzed the radio isotope composition of the produced methane to determine the relative contributions of each carbon source to methane production. The authors used this method to study the effect of low-temperature inhibition on the propionate degradation pathway. Single-carbon labeled acetate has been used extensively to study the relative activity of acetoclastic and SAO metabolisms in AD. Single-carbon labeled acetate produces unique isotope signatures in produced biogas depending on the relative activity of each pathway. This technique has been used to study methanogenic mechanisms under different temperatures (Karakashev et al., 2006; Nozhevnikova et al., 2007) and during ammonia-induced inhibition (Hao et al., 2015; Werner et al., 2014). Labeled substrate experiments have also been used in conjunction with other molecular methods to correlate different methanogenic pathways with specific organisms and communities (Hao et al., 2015; Ito et al., 2011; Mosbæk et al., 2016; Werner et al., 2014). Mulat et al. (2014) used membrane inlet quadrupole mass spectrometry (MIMS) and isotope labeled acetate to monitor the relative activity of acetoclastic and SAO pathways in near-real-time in a bench-scale reactor.

Naturally occurring isotope fractionation in biogas has also been used to estimate the relative importance of acetoclastic methanogenic and SAO pathways, however, it has typically been used in natural environments where the addition of large quantities of isotope labeled substrates is not practical. This approach is difficult to use because it requires knowledge of the isotope fractionation of naturally occurring acetate and system specific isotope fractionation factors (Conrad, 2005). In spite of these limitations, this method has been used in AD by comparing the relative fractionation of ^{13}C in biogas produced in uninhibited and inhibited bioreactors and inferring the naturally occurring isotope fractionation (Hao et al., 2011; Hao et al., 2017).

Stable isotopes can be combined with molecular techniques through DNA-, RNA-, and protein stable isotope probing (SIP) to reveal information about the phylogeny and activity of specific organisms responsible for the transformation of a particular substrate. DNA-SIP requires cellular growth for labelled elements to be incorporated into the DNA and subsequently detected (Lueders et al., 2016). Due to variations in density based on G-C content, labelled samples must be compared to unlabeled controls (Youngblut and Buckley, 2014). In AD, DNA-SIP has been used to identify the acetoclastic methanogens that dominate under high ammonia conditions (Hao et al., 2015) and identify cellulose degraders (Li et al., 2009; Limam et al., 2014). RNA-SIP, in contrast to DNA-SIP, can be used to track labels in both rRNA and mRNA and does not require cellular replication or growth (Lueders et al., 2016). Applied to AD, RNA-SIP has been used to trace labelled glucose through glucose-, propionate-, and acetate-degrading bacteria and acetoclastic methanogens (Ito et al., 2012; Ito et al., 2011) and reveal the diversity of fatty acid degrading bacteria (Hatamoto et al., 2007). Protein-SIP tracks label incorporation into proteins, providing information about cell activity as well as phylogeny (Jehmlich et al., 2010). To maximize the information obtained, it is best to combine protein-SIP with metagenomics (Jehmlich et al., 2010). This was done in AD to evaluate short-term changes resulting from high and low acetate concentrations (Mosbæk et al., 2016). These results revealed the importance of the SAO pathway under high acetate conditions (Mosbæk et al., 2016).

The fate of labeled substrates can be visualized using techniques such as microautoradiography (MAR) (Talbot et al., 2008) and nano-scale secondary ion mass spectrometry (NanoSIMS) (Musat et al., 2016). MAR involves the use of radioisotope labeled substrates and allows for visualization of actively metabolizing cells. Radioactive decay of the labeled substrate can be observed by the microbes that have taken up the substrate. Combined with fluorescence in situ hybridization (FISH), the actively metabolizing microbes can be identified. MAR-FISH has been used in anaerobic systems to identify novel acetate-utilizing bacteria (Ito et al., 2011), characterize the propionate oxidizing community (Ariesyady et al., 2007b), and identify low-abundance, highly active bacterial and archaeal populations (Ariesyady et al., 2007a; Ito et al., 2012). MAR-FISH is limited by radioisotope labeled substrates with suitable half-lives. While both organic substrates and carbon dioxide can be radiolabeled to target both heterotrophs and autotrophs, radioactive N (^{13}N) cannot be used because it has a very short half-life.

NanoSIMS overcomes the issues of radioisotope labeled substrates by using stable isotopes for visualization. NanoSIMS can also be combined with FISH (Chapleur et al., 2013) and has been used to visualize the spatial arrangement and isotopic enrichment of specific micro-organisms from AD (Li et al., 2008; Limam et al., 2014). NanoSIMS has also been combined with phylogenetic micro-arrays to measure isotopic enrichment of rRNA at much lower enrichment levels than traditionally

used for RNA-SIP (Mayali et al., 2012). Although not yet used to study AD inhibition, these powerful new molecular tools could help identify metabolic pathways most sensitive to specific inhibitors.

6. Real-time monitoring

6.1. Reporters

Fluorescent protein reporters are commonly used to identify the activity of specific proteins *in vivo*. A fluorescent protein is encoded onto a vector along with a protein of interest, and this vector is inserted into the microbes of interest. When the protein is expressed, the attached fluorescent marker is activated. Green fluorescent protein (GFP) is a widely used reporter protein, however GFP requires molecular oxygen in order to fluoresce, and is therefore not suitable for monitoring in anaerobic environments (Reid and Flynn, 1997). Anaerobic GFP (AnGFP) was developed to overcome this challenge and is capable of producing fluorescence in both aerobic and anaerobic environments (Drepper et al., 2007). AnGFP has been used to study microbes in the human gut (Landete et al., 2014) and lactic acid producing bacteria (Landete et al., 2015). Another reporter system used in anaerobic systems is the proprietary SNAP-tag™ system, which uses a modified protein derived from human DNA to tag a protein. The SNAP-tag™ protein then bonds with a fluorescent probe (Regoes and Hehl, 2005). SNAP-tag is suitable for anaerobic environments and has been used to label the nuclei of *Giardia* organisms (Regoes and Hehl, 2005) and to monitor the activity of specific pathogens in samples of dental plaque (Nicolle et al., 2010). These anaerobic-capable fluorescent probes allow *in vivo* studies of specific microorganisms in anaerobic cultures and could be used to study inhibition.

In addition to fluorescent reporters, other reporters can be used to track activity of specific organisms in anaerobic systems. Cheng et al. (2016) demonstrated a gas reporter system that uses the methyl halide transferase gene to produce a halogenated gas (e.g., CH_3F) when a specific gene is expressed. This gas is measured and correlated to the expression of the gene in question. The use of this system works in both aerobic and anaerobic environments, and thus is a promising reporter for use in AD systems. One potential drawback of using reporters is that they rely on engineered organisms that may be difficult to propagate in complex microbial communities such as those found in AD. Further, the reporters may constitute a metabolic burden for the host microorganism, and thus be lost or shed if present on a plasmid over generations of growth. Despite these limitations, reporters represent a powerful tool for providing information in near real-time about the activity of specific groups of microorganisms and their function in response to inhibitors.

6.2. MinION

The Oxford Nanopore MinION (MinION) sequencer is a small, portable, low-cost single-molecule sequencing device. The MinION platform is cost effective and capable of rapidly sequencing DNA and RNA. The rapid sequencing capability of the MinION may be used in real-time to increase understanding of reactor dynamics at greater temporal resolution. In the future, it may be possible to use this platform to perform rapid sequencing of microbial communities in bench-, pilot-, and full-scale AD systems, providing a near real-time profile of the microbial community and rapid identification of potential upset conditions in the reactor (e.g., by tracking abundance/activity of an indicator species).

Early reviews of the MinION indicated that it is capable of long read lengths but was prone to error rates as high as 38% (Laver et al., 2015; Mikheyev and Tin, 2014). According to a more recent review of the technology, improvements have resulted in a sequencing error rate of 8% (Ip et al., 2015). This error rate is still high compared with other next generation sequencing platforms. However, the Minion is capable of read lengths of 60 to 300 kbp compared with roughly 250 bp reads from other next generation sequencers. Researchers are hopeful improvements in chemistry and bioinformatics associated with the device will improve sequencing accuracy (Jain et al., 2016).

The platform has been used to characterize mixed microbial communities based on 16S rRNA gene sequencing in a mock microbial community with low diversity (Benitez-Paez et al., 2016), a mixed community from produced hydraulic fracturing wastewater (Ma et al., 2015), and the microbial community in a mouse gut (Shin et al., 2016). Karst et al. (2016) used MinION sequencing in conjunction with Illumina sequencing and a molecular tagging method to reduce error associated with the MinION sequencer when sequencing full-length 16S rRNA genes and SSU rRNA fragments collected from AD and several other complex environments. The method used by Karst et al. is primer independent and therefore reduces primer bias. The authors reported a significant increase in species diversity in anaerobic communities when compared with primer-dependent methods. With further development, MinION sequencing is likely to be an important molecular tool to characterize AD inhibition in near real-time.

7. Mitigation strategies

Inhibition can reduce energy recovery or necessitate reseeding under extreme instances, negatively impacting the economic favorability of AD systems. New molecular methods have elucidated many inhibition mechanisms in AD and evaluated how common inhibitors impact microbial community structure and activity. Various mitigation strategies have been studied to reduce inhibitory effects on the AD microbiome. For example, bioaugmentation, the addition of key enriched cultures, has been applied as a strategy to increase performance of AD systems and decrease sensitivity to inhibitors. However, there are mixed reports on the effectiveness of bioaugmentation, as reviewed by De Vrieze and Verstraete (2016). A study that bioaugmented via addition of a methanogenic propionate degrading community (0.3 g dry cell weight L^{-1} d^{-1}) at high ammonia stress conditions (3.0 g N L^{-1}), reported that methane recovery rate increased by 21% and propionic acid degradation increased by 51% after 45 days, compared to a non-bioaugmented reactor (Li et al., 2017b). The increased performance was partly attributed to enrichment of *Methanosaetaceae*, the most abundant methanogenic population in the bioaugmentation culture (> 90% relative abundance). Further, recovery of the non-bioaugmented reactor after near failure (almost no methane production after 75 days) was demonstrated by routinely adding a double dosage (0.6 g dry cell weight L^{-1} d^{-1}) of the bioaugmentation culture. Similarly, another study reported that bioaugmentation of *Methanoculleus bourgensis* MS2 in a CSTR with elevated ammonia concentration of 5 g NH_3 L^{-1} led to a 31.3% increase in methane production compared to a non-bioaugmented reactor (Fotidis et al., 2014). Relative abundance of *Methanoculleus* increased by 5-fold after 39 days after bioaugmentation, suggesting that the bioaugmented culture was functionally active in the CSTR. An earlier study by the same authors, however, reported that bioaugmentation of an ammonia tolerant SAOB co-culture, *Clostridium ultunense* spp. nov., and *Methanoculleus* spp. strain MAB1 in a UASB reactor subjected to ammonia stress, did not prevent system failure (Fotidis et al., 2013). The authors hypothesized that slow growth of methanogens in the co-culture limited success of these experiments. Another study that tested bioaugmentation to mitigate ammonia inhibition was similarly unsuccessful (Westerholm et al., 2012).

An alternative strategy to prevent inhibition in AD is temporal acclimation to inhibitors which can result in microbial community adaptation (Dai et al., 2016; Gao et al., 2015; Silva et al., 2014; Silvestre et al., 2011). A study that used this approach for digestion of a protein-rich substrate in a CSTR, reported that *in situ* acclimation led to tolerance to ammonia concentrations of up to 4.2 g L^{-1}, where relative abundance of *Firmicutes* and hydrogenotrophic methanogens increased in response to elevated ammonia (Gao et al., 2015). Another study that compared bioaugmentation and acclimation as a strategy to decrease

inhibition of LCFA showed that long-term acclimation (> 100 days) by applying an increasing load of oleate resulted in reduced lag phase in biogas production, whereas bioaugmentation of a co-culture of *Syntrophomonas zehnderi* and *Methanobacterium formicicum* did not have any significant impact (Silva et al., 2014). Co-digestion of different substrates has also been suggested by some studies as a relatively simple method to dilute substrate feed that might contain inhibitors (Astals et al., 2014; Pagés-Díaz et al., 2014). For example, a study that used slaughterhouse waste feed reported that dilution of inhibitory compounds with co-digestion led to improved methane yield compared to mono-digestion reactors (Astals et al., 2014). Other methods for inhibition mitigation include thermal pre-treatment (Ennouri et al., 2016), alkaline pre-treatment (Koyama et al., 2017), and enzyme addition (Meng et al., 2017). A study that evaluated the addition of three lipases to hydrolyze food waste rich in crude lipid revealed that using two of the lipases increased methane production by 81–158% in animal fat, 27–54% in vegetable oil, and 37–41% in floatable grease waste digestions (Meng et al., 2017).

Electrically conductive support media could also be an approach to enhance performance in AD systems via DIET, an electron exchange mechanism that does not require diffusive molecules (H_2 and formate) for electron transfer (Summers et al., 2010). Researchers have thus considered its application as a strategy to increase performance (Kato et al., 2012; Lin et al., 2017) and potentially mitigate inhibition. DIET can occur through biosynthesized nanowires and pili or through addition of a semi-conductive compound such as activated carbon (Liu et al., 2012), biochar (Chen et al., 2014c), nano-magnetite (Jing et al., 2017), or graphene (Lin et al., 2017). DIET has the potential to enhance performance because traditional electron transfer between syntrophic bacteria and methanogens can be rate limiting in AD (Stams, 1994). It has been demonstrated that both *Methanosarcina* (Rotaru et al., 2014a) and *Methanosaeta* (Rotaru et al., 2014b) can receive electrons via DIET. Various semi-conductive minerals have been shown to facilitate electron transfer in mineral-based DIET. For example, supplying haematite or magnetite resulted in an increased abundance of *Geobacter* spp., a common exoelectrogen, in a study that used rice paddy field soil to enrich methanogens (Kato et al., 2012). The study noted that when methanogenic inhibitors were added, the growth of *Geobacter* spp. also declined, suggesting that *Geobacter* only grew in syntrophy with methanogens (Kato et al., 2012). Further, the study reported that the supplementation of the iron oxides resulted in faster methane production rate and reduced lag phase in methanogenesis (Kato et al., 2012). Similarly, the use of graphene nanomaterials resulted in a 25% increase in methane yield (Lin et al., 2017). Theoretical calculations indicated that DIET facilitated higher electron transfer flux than was achievable by electron transfer via diffusive molecules. Microbial community analysis revealed *Geobacter* and *Pseudomonas* as electron donors and *Methanobacterium* and *Methanospirillum* as possible electron receivers. Another study demonstrated mineral based DIET using carbon cloth in co-cultures of *Geobacter metallireducens* and *Methanosarcina barkeri* (Chen et al., 2014b). The use of mutant *Geobacter metallireducens* strains in the co-culture, lacking electrically conductive pili or pili associated cytochromes, facilitated the distinction from pili-based DIET. Supplying AD with semi-conductive minerals could be an effective strategy to increase the efficiency of methanogenesis, but further studies need to be conducted to confirm the prospects of using DIET for AD inhibition prevention.

8. Conclusion

Researchers are beginning to use a diverse set of molecular tools to elucidate microbial community interactions in AD systems to better understand and devise strategies to prevent inhibition. The AD community must continue to develop and employ advanced molecular tools to gain a mechanistic understanding of how inhibitors influence stability while also standardizing sequencing methods and better metadata reporting to facilitate cross-study analysis. Techniques that map substrate consumption and offer real-time feedback could provide breakthroughs in inhibition prevention. It is important that we couple these advanced tools with hypothesis-driven research to improve resiliency and broaden implementation of AD systems.

Acknowledgements

YMA was supported by a Provost Fellowship from the University of Southern California and the National Science Foundation under Grant No. CBET-1605715. MZA was supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie project MicroArctic under grant agreement No. 675546. AMB was supported by a fellowship from the Rice University Civil and Environmental Engineering Department.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.08.210.

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