

Bioengineering options and strategies for the optimisation of anaerobic digestion processes

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Anaerobic digestion (AD) is a complex biological process and the microbial diversity and dynamics within the reactor needs to be understood and considered when process optimisation is sought after. Microbial interactions such as competition, mutualism, antagonism and syntrophism affect the function and the survival of single species in the community; hence they need to be understood for process improvement. Although the relationship between process performance and microbial community structure is well established, changes in the community might occur without detectable changes in gas production and reactor performance. Recent molecular based studies have highlighted the complexity of AD systems revealing the presence of several uncultivated species and the need for further research in this area. However, this information is still rarely used for process optimisation. The integration of next generation sequencing technologies, such as 454-pyrosequencing, with other techniques, such as phospholipid-derived fatty acids analysis can provide an holistic understanding of the microbial community. In addition, the in depth phylogenetic resolution provided can aid environmental ecologists and engineers to better understand and optimise AD process and consolidate the information collected to date.

Keywords: Anaerobic digestion; microbial diversity; process optimisation; bioaugmentation; microbial ecology.

Introduction

Anaerobic Digestion (AD) is the biological conversion, in the absence of oxygen, of organic waste into biogas (comprising methane and carbon dioxide). The AD process is an attractive waste management strategy as it has a number of useful outputs, including biogas, heat and digestate [1-4]. The use of the anaerobic process to treat wastewater sludge solids and high-strength organic wastes is well established. However, in the past decade the need to divert wastes from landfill, the requirement for the generation of renewable energy, and the requirement to reduce greenhouse gas emissions has led to the

application of anaerobic process to a wide variety of new wastes, including food wastes, municipal solid wastes (MSW), distillery wastes, farm wastes, and slaughter house wastes [1, 5-10]. Although AD is an established technology the process is often run well below its full potential and optimisation of this technology is still required, particularly in the context of digesting new feedstock types [5, 11].

The optimisation of the AD process has mainly been focused on the operational parameters such as reactor configuration, mixing, temperature, feedstock composition and pre-treatment of wastes [5, 11-21]. For example, co-digestion of different waste material, which has a number of potential benefits in AD including improving the overall availability of nutrients and the dilution of inhibitory compounds, has been effective in improving AD of new waste streams [22-28]. Co-digestion of algal sludge with waste paper and co-digestion of cattle slurry with vegetable wastes and chicken manure have both been shown to result in a doubling of methane yields [29, 30]. However, co-digestion has also been shown to cause changes in the microbial dynamics in AD [31-33]. Although tools have been developed to optimise co-digestion based on AD operational performance and parameters, this has still to be done to identify and optimise the microbial communities involved in the process [34].

AD is a biological process therefore it is also important to understand the microbial diversity and dynamics within the digesters. It is well known that factors such as mixing, feedstock composition, and OLR/HRT can influence the structure and dynamics of the microbial community in AD [31-33, 35-41]. In contrast it is less well known how the structure of the microbial community influences AD performance as changes in microbial community structure can occur without detectable changes in gas production and reactor performance [42-47]. It has been suggested that high functional redundancy and microbial population variation between digesters, particularly in the

bacterial populations, negates any clear and/or repeatable trend between performance and bacterial community structure [43, 44, 48]. However, clear relationships between the less diverse archaeal populations and the community structure have been suggested, with *Methanosepta* considered as an indicator of good system health while a shift to *Methanosarcina* could indicate periods of instability in terms of methane production [38, 49, 50]. It is possible that the lower diversity of the archaeal community, in comparison to the bacterial community, eases the understanding of the relationships between performance and community structure. However a more detailed analysis of the bacterial community can further help to understand the relationships between AD performance and bacterial community structure.

Advances in culture independent microbiology over the last 20 years, and in particular next generation sequencing (NGS) allow to examine AD microbial communities in far greater depth than previously possible [51-54]. This represents an opportunity to develop a deeper understanding of the relationships between AD performance and microbial community structure and function. This review seeks to highlight the value and potential of applying knowledge on the microbial communities involved in AD to achieve process optimisation.

Overview of the microbial ecology in AD processes

The anaerobic digestion process includes three main conversion steps carried out by the Bacteria, hydrolysis, acidogenesis, and acetogenesis, and one conversion step, methanogenesis, carried out by the archaea [55, 56]. Disturbances at one stage have downstream effects on the other populations that often cause an imbalance in the process. This can result in the accumulation of intermediate products, indicating that the microbial community is under stress. An imbalance of the conversion products between the acid forming stages and the methanogen stage can cause an increase of volatile fatty

acids (VFA) and a drop in pH [57]. Most of the methane-forming archaea are active at pH values between 6.8 and 7.2 [50, 58]. If pH values in the reactor drop below this range, the archaea will be outcompeted by the fermentative bacteria which will continue to produce volatile fatty acids further lowering the pH. In this condition, acetic acid is metabolised through other pathways such as hydrogen production or sulphate reduction and therefore low methane production is reported in digesters [59-65]. A theoretical representation of how a microbial community within AD may respond to perturbation is shown in Figure 1. A community can either demonstrate resistance (remain the same), resilience (change and return to original state) or adaptation and resulting in either unchanged or improved functionality in performance parameters such as methane production. The factors that influence these outcomes are at present unclear but likely to be related to the magnitude and duration as well as the type of the perturbation applied (pH change, chemical inhibition or temperature for example). The outcomes will also be influenced by the initial microbial community. The key features of the microbial community that will play a significant role are yet to be investigated but could include the existence of syntrophic relationships, the functional characteristics of the individual species, overall species diversity in the community and the distribution of diversity across the community (evenness). By gaining a better understanding of the factors that control the outcomes indicated in Figure 1, environmental microbiologists, engineers and operators will be able to better predict AD performance and therefore to optimise and control the process. A consolidation of the current knowledge of the diversity present in AD, its roles, and how the physico-chemical parameters affect them is therefore required to develop microbial optimisation of AD.

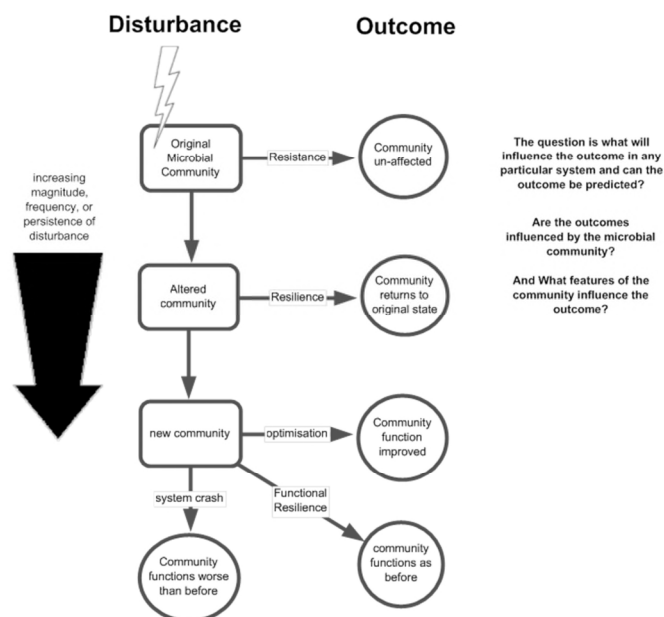


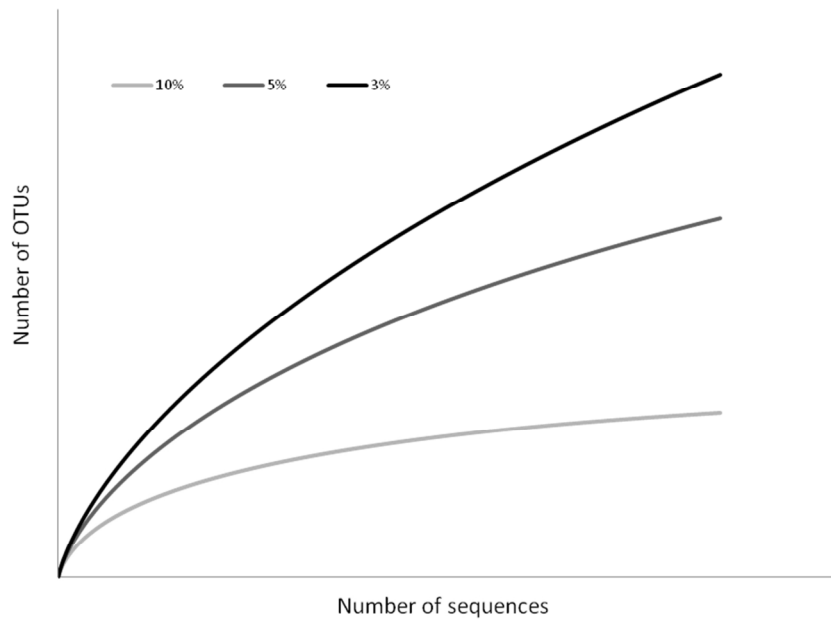
Figure 1. Theoretical response of a community to disturbance adapted from Allison and Martiny [174].

Microbial diversity of anaerobic digestion

Retrieval of sequences from NCBI

To summarise the diversity present in AD a set of sequences was retrieved from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) using the search term “anaerobic digester”. Sequences under 200 base pairs and those not originating from studies of AD were removed. A total of 3457 bacterial and 2946 archaeal sequences were retrieved. These sequences were aligned and clustered with the Ribosomal Data Project (RDP) pyrosequencing pipeline (<http://pyro.cme.msu.edu/>) and then classified using the RDP Naive Bayesian rRNA Classifier, (Version 2.5 01/05/12, Taxonomical Hierarchy: RDP 16S rRNA training set 9, Submission Date: 03 Oct 2012) using the default confidence threshold of 80 % to ensure good phylogenetic resolution of all OTUs [66]. Rarefaction analysis (Figure 2) shows that at 5 % phylogenetic

distance most of the diversity had been sampled whereas at 10 % phylogenetic distance



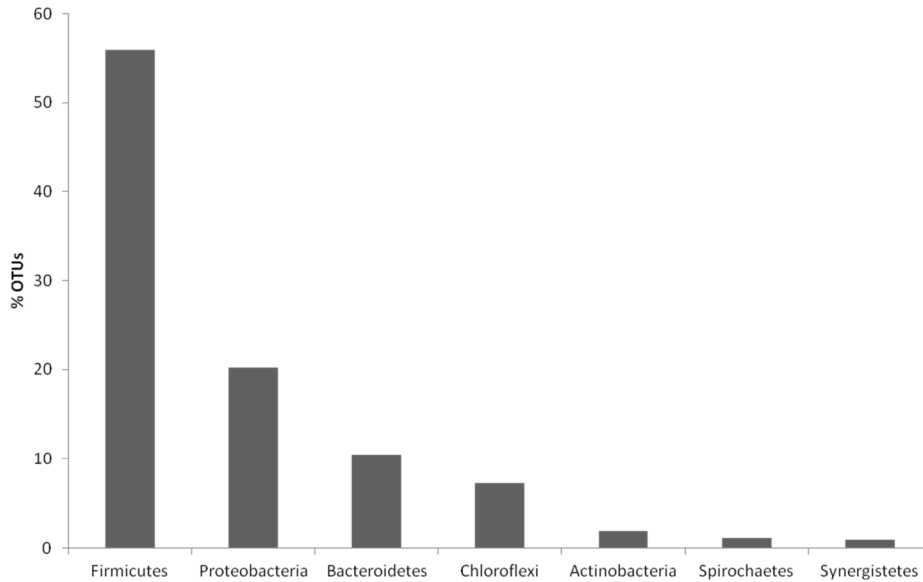
saturation had been reached.

Figure 2. Rarefaction curve of OTUs identified using Ribosomal Data Project (RDP) pyrosequencing pipeline per-sequences sampled at 3, 5, and 10 % phylogenetic distance.

1.3.2. Bacterial diversity in AD

The bacterial phylum *Firmicutes*, accounted for 1393 sequences. Of these 841 were attributed to the class *Clostridia* and 233 to *Bacilli*. Other phyla included *Proteobacteria* (524 sequences) *Bacteroidetes* (266 sequences) *Chloroflexi* (81 sequences) and *Actinobacteria* (51 sequences) (Figure 3). Twenty-five sequences were identified for both *Spirochaetes* and *Synergistetes*. Other phyla identified included *Thermotogae*, *Tenericutes*, *Lentisphaerae*, *Armatimonadetes*, *Acidobacteria*, *Chlorobi*, *Deinococcus-Thermus*, *Planctomycetes*, *Fusobacteria*, *Caldiserica*, *Nitrospira*, *Verrucomicrobia* and *Fibrobacteres* which all had less than 10 sequences. The ability to classify and list the diversity of the microbial communities in AD has increased with the

advent of NGS technologies. However without an understanding of the roles of these groups and how they respond to changes in the physicochemical parameters it remains



difficult to optimise AD processes.

Figure 3. Distribution of bacterial sequences from anaerobic digesters at Phylum level.

The archaeal diversity in AD

Methanogenesis is the final stage of AD and is carried out exclusively by methanogenic archaea belonging to the phylum *Euryarchaeota*. There are five orders of

Euryarchaeota that can carry out methanogenesis comprising *Methanopyrales*,

Methanococcales, *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*.

All of them are obligate methane producers that derive most or all of their energy from methanogenesis [58, 67]. An analysis of the archaeal diversity present in anaerobic

digesters based on sequences retrieved from the NCBI Figure 4 showed that only the

orders *Methanosarcinales* (1514 sequences) *Methanomicrobiales* (504 sequences) and

Methanobacteriales (246 sequences) are predominant in AD systems. The methanogens

feature a limited metabolic diversity with only three main pathways of methane

production including the hydrogenotrophic, acetoclastic, and methylotrophic pathways. The hydrogenotrophic pathway is common to almost all methanogens while the acetoclastic and the methylotrophic pathways are restricted to the *Methanosarcinales* [68, 69]. Archaea are less diverse, metabolically slower and less resilient to stress than the bacterial component of the community in AD. Methanogenesis is therefore often considered more susceptible to stress and instability than the other stages [57, 69-72]. In the following section the effect of parameters such as feedstock and VFA concentration on both the bacterial and archaeal communities are examined.

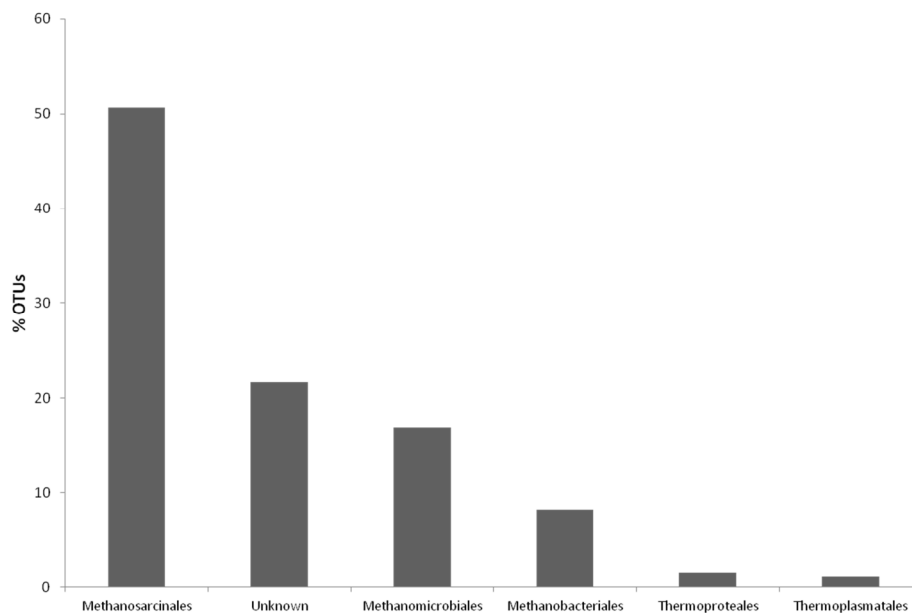


Figure 4. Distribution of methanogenic sequences from anaerobic digesters at Order level. 682 sequences were unclassified.

Influence of physicochemical parameters on microbial communities in AD

Effect of feedstock on microbial community

Bacteria are responsible for the first three stages of AD (hydrolysis, acidogenesis and acetogenesis) and as a result of this, they directly interact with the feedstock

composition. This is in contrast with the archaea which are only able to convert the products of the final bacterial stages into methane. Therefore it would be expected that the structure of the bacterial community, and in particular the hydrolytic bacteria, would be heavily influenced by the feedstock characteristics. Indeed, several studies have shown that feedstock affects the bacterial community structure [31, 33, 73-75]. Most of the observed changes are within the hydrolytic groups (*Clostridiales* and *Bacteroidetes* Orders). However, the previous generation of culture independent microbiological techniques were biased towards the dominant community members. As mentioned in section 1.3.2 *Clostridia* and *Bacteroidetes* were the most common sequences in AD studies, it is therefore possible that the results were biased to these bacterial groups and that studies with a higher level of phylogenetic resolution will reveal less dominant but feedstock specific degrading-members. Table 1 summarises the dominant bacterial taxa retrieved in NCBI and their possible roles in AD. *Clostridia* are dominant in digesters with high cellulose content [53, 73, 76] whereas *Bacteroidetes* are prevalent in digesters fed with protein rich feedstock such as bovine serum albumin [77] distillers grains [78] and casein [79]. The *Deltaproteobacteria* and *Actinobacteria* are associated with the digestion of lipid rich wastes and are involved in the beta-oxidation of long chain fatty acids (LCFA) [80-82].

The relationship between microbial community and the feedstock is an important factor in AD optimisation, particularly as AD expands to new feedstocks and co-digestion substrates. Changes in feedstock and co-digestion substrate can influence the microbial communities of the digesters and have subsequent consequences on the methane yields and the digesters stability. Such issues need to be understood to ensure optimal AD performance. The core populations needed for the optimal digestion of

different feedstocks need to be identified so that AD operators can ensure optimal conditions for AD process.

Table 1. Summary of the bacterial Phyla and Classes associated with feedstock type in anaerobic digestion.

Phyla	Class	OTUs	Main Possible Roles	References
<i>Firmicutes</i>	<i>Clostridia</i>	1393	hydrolytic (cellulose)	[53, 76, 150,
			acetogenesis	151]
	<i>Bacilli</i>	233	acetogenesis	[152]
<i>Proteobacteria</i>	<i>α-proteobacteria</i>	23	acetogenesis	[54, 80, 91, 153, 154]
	<i>β-proteobacteria</i>	108	acetogenesis	
	<i>δ-proteobacteria</i> ,	159	acetogenesis and LCFA oxidation	
	<i>γ-proteobacteria</i>	158	acetogenesis	
	<i>ε-proteobacteria</i>	67	acetogenesis	
<i>Bacteroidetes</i>	<i>Bacteroidia</i>	142	protein hydrolysis and amino acid	[77-79]
			fermentation	
<i>Chloroflexi</i>	<i>Anaerolineae</i>	81	syntrophic LCFA oxidation of VFA	[155-158]
<i>Actinobacteria</i>		51	LCFA oxidation and digester	[159-163]
			foaming	
<i>Synergistetes</i>		25	amino acid fermentation syntrophic	[164-167]
			acetogenesis	

Effects of organic loading rate (OLR) on the microbial community in AD

OLR is a key parameter for AD operators as higher OLR corresponds to a greater amount of wastes processed. The OLR clearly affects the bacterial community present in AD as increase in OLR has been shown to change the amount and composition of VFA produced by the acidogenic bacteria and therefore influencing the metabolic function of the bacterial community [83]. Rincón et al. [38] showed that increasing the OLR from 0.7 to 9.1 kg VS m⁻³ day⁻¹ resulted in a greater bacterial diversity with a shift

from a *Clostridium* dominated community to a community comprising members of *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Deferribacteres*. Krakat et al. [84] also observed relationships between OLR and bacterial community structure with an increase of *Acidobacteria* and *Chloroflexi* (> 65 % of clones) at the highest OLR (13 VS m⁻³ day⁻¹) and a decrease of *Planctomycetes*, *Alcaligenaceae*.

In regards to OLR effect on the methanogens, contrasting results have been reported. For example, Rincón et al. [38] reported dominance of *Methanosaeta* at OLR ranging from 0.7–9.1 kg VS m⁻³ day⁻¹ and Gomez et al. [40] also reported no change in an archaeal community comprising *Methanomicrobiales*, *Methanosarcinales* and *Methanobacteriales* at OLR ranging from 3.4 to 5.0 kg VS m⁻³ day⁻¹. In contrast, Montero et al. [85] observed an increase in *Methanosaeta* (acetotrophic methanogens) from 1 % to 30 % as OLR was increased from 4.4 to 7.2 kg VS m⁻³ day⁻¹ and a corresponding decrease of *Methanobacteriaceae* from 11 % to 7 % (hydrogenotrophic methanogens). Further to this, Lerm et al. [86] recently showed a reverse relationship with a switch from *Methanosarcina* to the exclusively hydrogenotrophic methanogens *Methanospirillum* and *Methanoculleus* as OLR was increased from 2.5 to 40 kg VS m⁻³ day⁻¹. The archaeal shift observed by Lerm et al. [86] can be related to a significant increase in the VFA concentration and/or an organic overload of the digesters suggesting that OLR only affects the archaeal community if it results in changes in other parameters such as VFA concentration and pH.

Overall the studies discussed here on the influence of OLR on the Bacteria and Archaea involved in AD highlighted contrasting effects and therefore the difficulty of developing a predictive understanding of the relationship between digester performance and microbial community structure and dynamics.

Effects of VFA composition and concentration on microbial community

VFA are intermediate products produced during acidogenesis and acetogenesis. Acetic acid is the key substrate for methanogenesis, but if its production rate is faster than its utilisation rate by the methanogens, digester instability will occur. For example, shift in the archaeal population from *Methanosaeta* to *Methanosarcina* in digesters experiencing a VFA increase from to more than 1.5 g l⁻¹ has been observed by several authors [87-90]. Hori et al [57] also showed that VFA accumulation resulted in a 10,000 fold increase in the gene expression for the hydrogenotrophic methanogen *Methanothermobacter* and Delbès et al. [70] reported the dominance of *Methanobacterium* at high acetic acid concentrations (> 3 g/l). Analysis with fluorescence in situ hybridization (FISH) indicated that syntrophic interactions between hydrogenotrophic methanogens and bacteria is key in the degradation of VFA and recovery of digesters [57]. McMahon et al. [91] further showed that digesters with a history of poor performance had proportionally higher numbers of *Methanosarcina* than *Methanosaeta* as well as a higher number of syntrophic bacteria including *Syntrophobacter*, *Smithella*, and *Syntrophomonadaceae*, and consequently that the microbial community of the digesters was more tolerant to VFA accumulation as a result of the high organic loading. These results demonstrate that the structure of the microbial community can positively influence AD performance. Clearly higher numbers of syntrophic Archaea and bacteria are desirable for AD which can be increased by past stress in the reactor. This can be related to Figure 1 as a perturbation altering the community structure and resulting in optimised performance.

Although the relationship between the VFA concentration and the archaeal community dynamics are well understood, our understanding of the relationship between the bacterial community dynamics and the VFA composition and concentration

is still limited. A relationship between acetic acid concentration and *Clostridia* abundance was proposed by Delbès et al [70]. However in a previous study, Delbès et al. [92] reported no difference in the bacterial response to acetate, propionate, or butyrate suggesting that quantifying the total concentration of VFA was more important than reporting the concentration of speciated VFA. This finding is somewhat surprising as bacteria are the VFA producers in AD and therefore likely to influence the VFA composition and concentration in AD systems. Also it has been demonstrated that propionic acid has a higher inhibition effect than acetic and butyric acids on methane production [64, 93]. This controversial finding reinforces the need to further investigate the relationship between specific archaeal/bacterial groups and specific VFA.

Effect of ammonia on the microbial community

Many farm wastes including pig slurries, slaughter wastes, cattle and poultry manure have high concentration of ammonia which can be either inhibitory or beneficial to maintain optimal AD process [94-99]. Several studies have reported significant increase of hydrogenotrophic methanogens belonging to *Methanosarcinaceae*, and to a less extent to *Methanomicrobiales* and *Methanobacteriales* in digesters with ammonia concentration of $> 3 \text{ g l}^{-1}$ [87, 100, 101]. The formation of multicellular units by *Methanosarcinaceae* at high concentrations of ammonia protects them and also results in more efficient syntrophic relationships between methanogens and bacteria for interspecies hydrogen transfer. The hydrogenotrophic methanogens are therefore favoured under this condition instead of the acetotrophic methanogens [100, 102]. This can be related to Figure 1 as an example of a perturbation (increase in ammonia concentration) causing a shift in the microbial community structure and function (switch to hydrogenotrophic methanogenesis) which can preserve AD function.

In contrast, the effect of ammonia on the bacteria is unclear and results suggest that the methanogens are inhibited well before ammonia affects the bacteria [103]. Koster and Lettinga [104] showed that the production of VFA by bacteria is not significantly affected by ammonia concentration. They also demonstrated that after exposing digesters to ammonia concentration up to 9 g l^{-1} for three weeks, ammonia tolerance by the methanogens was improved by 6 times. This finding suggests that the microbial community of digesters can be easily optimised to produce methane from feedstocks with high ammonia. This can be related to Figure 1 as a disturbance (high ammonia) resulting in an optimised community. To date, while the mechanism of this adaption is not fully understood, the predominance of the hydrogenotrophic methanogens and the favoured syntrophic interactions with the bacteria at high ammonia concentration suggest that the acclimation of the community to high VFA concentrations is related to changes in the community structure.

Effect of trace metals on AD microbial communities

The availability of trace metals in AD and effect on performance has been a major topic of research for over 30 years [15, 105-109]. Additions of trace metals such as cobalt, molybdenum, iron, nickel selenium, and sulphate have been shown to improve methane yields [107, 110-114], improve stability [115-119], and optimise long-term AD performance [120, 121] largely through reducing accumulation of VFA. It is known that trace metals such as cobalt, nickel, iron, zinc, molybdenum, and tungsten are important for the activity of the enzymes involved in methane production in AD [122, 123]. Despite this the effect of trace metal concentration and addition on the structure of the microbial community structure has not been extensively researched. Feroso et al. [124] observed decrease in numbers of *Methanosarcina* and associated decrease in performance parameters under cobalt limited conditions in a UASB reactor treating

methanol, results also suggested cobalt addition may be a suitable strategy for recovering *Methanosarcina* populations. Banks et al. [121] showed that the dominant methanogenic populations in digesters with high ammonia concentration (4-6 g l⁻¹ TAN) and varying trace metals concentrations were *Methanoimicrobiales* indicating that ammonia concentration was a more important factor than trace metals in structuring the methanogenic community. Feng et al [48] investigated the effect of additions of cobalt alone, a combination of nickel/molybdenum/boron, or a combination of selenium/tungsten on microbial community structure and AD performance. The best methane production was related to high selenium and tungsten concentration with low cobalt. Trace metals concentration did not influence the relative abundance of the most dominant bacterial population (*Actinobacteria*) but two bacterial populations both related to *Firmicutes* were positively correlated with the nickel/molybdenum/boron treatment alone and negatively correlated with nickel/molybdenum/boron combined with cobalt. The archaeal populations showed a much greater correlation with the trace metals with a *Methanoculleus* population positively correlated with selenium and tungsten alone but negatively correlated with nickel/molybdenum/boron and selenium and tungsten when they were supplemented together. In contrast *Methanosarcina* population was positively correlated with the nickel/molybdenum/boron treatment. Feng et al. [48] demonstrated that trace metals influence the structure of the bacterial and archaeal populations in AD, however the response of archaeal populations from the same genus differed, and when nickel/molybdenum/boron was used in combination with cobalt the correlation was reversed. This shows that the relationship between microbial populations in AD and trace metals is complicated and that different combinations of trace metal supplementations can have antagonistic effects. As it is clear that trace metal concentration is a key parameter in optimizing AD more research

is required to understand the influence of trace metals on the microbial community to fully exploit this knowledge to optimise AD.

Microbial optimisation of AD

Bioaugmentation for AD optimisation

Bioaugmentation with a particular species or consortium of species could allow plant operations to change the existing microbial community so that it is optimised to carry out a specific function [125, 126]. Bioaugmentation has been used for the remediation of contaminated soils and ground waters and has also been applied extensively to aerobic wastewater treatment [125-133]. Bioaugmentation has been also applied towards the optimisation of a number of aspects of anaerobic digestion including degradation of problematic feedstocks with high cellulose or lipid content, improvement of recovery from perturbation, and faster start-up times (Table 2). However bioaugmentation is not always successful and further research is required to develop bioaugmentation as an optimisation strategy in AD [134]. The effect of bioaugmentation on the indigenous community needs to be examined as interactions such as predation and competition may result in negative effects on the community rather than improved performance. Additionally the survival and integration of the exogenous population into the reactor needs to be examined to establish how to maintain the effect of bioaugmentation over long periods. Perhaps the most important question to address is what species/cultures are going to have a beneficial effect on the community. To answer this question, further research identifying novel species with beneficial physiological traits, as reported by [135, 136] will be useful. Another important approach will be the analysis of microbial communities in digesters under specific conditions, such as recovering from overload, so that an understanding of what

type of community consortium will be desirable for a certain situation can be developed.

Table 2. Summary of successful bioaugmentation studies in AD.

Optimisation	substrate	Microorganism/s	Benefits	Reference
		Hemicellulose		
	Cattle manure	degrading bacterium	+ 30 % CH ₄ potential	[17]
	biofibers	<i>Caldicellusiruptor</i> and <i>Dictyoglomus</i>	+ 10-24 % CH ₄ yield	[168]
Feedstock	poultry litter	<i>Clostridium cellulolyticum</i> , <i>and thermocellum</i> , <i>Caldicellulosiruptor</i> <i>saccharolyticum</i>	Up to 15 % increase in CH ₄ production	[169]
	Lipid rich waste	<i>Syntrophomonas zehnderi</i>	Improved CH ₄ production rate	[170]
		<i>Clostridium lundense</i>	Improved CH ₄ production rate	[171]
Faster recovery	Oxygen exposure	<i>Methanosaeta</i> , <i>Methanoculleus</i> , and <i>Methanospirillum</i>	70-80 days faster recovery	[130]
	Organic overload	a propionate-degrading enrichment culture	25 days faster recovery	[172]
Improve reactor start-up	pharmaceutical effluent	Anaerobic sludge from plant treating antibiotic effluent	faster reactor start-up time	[173]
Improved resilience	Low pH high VFA	acid tolerant <i>Methanobrevibacter</i>	+ 7-12 % CH ₄ production	[135]

Manipulation of process and AD design for microbial optimisation

An alternative to bioaugmentation is to promote microbial community diversity by changing the operational conditions of AD. Research has shown that digesters with greater flexibility in microbial community structure are more resilient to perturbation than more stable communities [42, 137]. Hashsham et al. [137] showed that digesters that were able to process feed through a network of multiple routes in parallel were more stable than those that processed feed through sequential pathways. Therefore promoting functional diversity in the microbial community is one possibility for improving AD stability. This has been recognised and incorporated into the design of AD systems such as baffled digesters or membrane reactors. Functional diversity can also be promoted by using granular substrates [138]. It was suggested by Briones & Raskin [138] that incorporating changes in operational conditions such as modifying the OLR can also enhance the functional diversity and performance of the digesters. This was also proposed by McMahon et al. (2004). More recently Palatsi et al. [139] showed that digesters exposed to repeated LCFA pulses had faster recovery times. Therefore there is mounting evidence that the resilience of AD microbial communities can be enhanced through manipulation of the operational conditions which can be subsequently used to optimise AD process.

Microbial community monitoring as decision support tool for AD performance

The lack of reliable sensory equipment and control systems have been reported as one of the major reasons for AD not being operated at optimal conditions [11]. This was stated in the context of monitoring the biochemical process, however as demonstrated in this review the microbial community must be considered in AD optimisation. Talbot et

al [51] in a review of nucleic acid based techniques to characterise communities in AD systems point to the development of laboratory-on-chip systems for eventual on-line monitoring of bioreactors. This technology has already been demonstrated for fast characterization the human gut microbiota, where similar microbial consortium are found [140]. Microarray chips have also been successful in characterising the archaeal community in anaerobic sludge, and therefore it is appropriate to think that an accurate, viable and cheap method for monitoring microbial communities in AD will be available soon [141].

The development of culture independent techniques such as phospholipids analysis (PLFA), denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) and others for microbial community fingerprinting has undoubtedly improved our understanding of microbial communities in AD [44, 92, 100, 102, 142]. Fingerprinting techniques, such as DGGE have proved more effective in studying the less diverse archaeal community than the highly diverse bacterial community. This is due to the relative complexity of the bacterial community which is therefore much more challenging to characterise. Community fingerprinting has allowed microbiologists to look at community shifts in relation to changes in the physicochemical parameters, such as VFA profiles, in AD. The application of the so-called next generation sequencing technologies, such as 454-pyrosequencing, represents one of the most exciting areas of development in AD. Combining 454-pyrosequencing to phylogenetic microarray has provided a cost effective way of obtaining high-resolution data on microbial community structures and function in AD. Schlüter et al. [53] and Kröber et al. [52] analysed the microbial communities in a production scale AD plant fed with maize silage, green rye and liquid manure. The data obtained in these studies far surpasses the depth of information gained in previous studies based on

fingerprinting techniques. For example the identification of less dominant members such as *Syntrophobacterales* and *Synergistia* have been shown to have greater correlation with the changes in performance observed [54]. As NGS technologies become cheaper and the strategies for analysing the data get more refined, our understanding of the structure and function of microbial communities, such as those found in AD reactors will improve exponentially. However, these techniques still provide limited quantitative information. To this end, analysis of the lipid content of the community including phospholipid-derived fatty acids (PLFA) for bacteria and phospholipids etherlipid (PLEL) for archaea can reveal changes in biomass and function of microbial communities [142-147]. In particular, Schwarzenauer and Illmer [147] showed that monitoring PLFA could identify changes in the microbial community associated with changes in AD performance. The relative cheapness of lipid fingerprinting and the use of high throughput PLFA techniques, developed by Buyer and Sasser [148], make it possible to monitor changes in biomass and lipid structure over long time series, at both lab and potentially full scale operational AD plants. Lipid fingerprinting, which are overlooked by many microbiologists outside soil science, can represent a valuable tool in AD optimisation.

Conclusions and research gaps

The preceding sections of this review have summarised some of the recent developments in microbial ecology in AD. Culture independent analysis of communities has improved our understanding of the process but even so there is no obvious direct application of this information to deliver significant process optimisation in AD. Industry remains sceptical to the benefit that microbial optimisation can provide and this will remain the case until the benefits of microbial optimisation can be empirically proved. There is a perception that the microbial communities are so diverse that it will

not be possible to produce a clear understanding of the role of the microbial community. This perception is supported by research showing highly dynamic communities in stable reactors which gives weight to the argument that community shifts are not related to performance [43]. As reported in this review there is a great deal of work demonstrating that the methanogenic community is influenced by the concentrations of intermediate products such as VFA and other inhibitory compounds such as ammonia. The factors that influence the bacterial community are less understood, but the improved resolution of NGS in combination with other techniques such as lipid fingerprinting may help to improve our understanding of this aspect of the AD community. McMahon et al. [149] called for the integration of microbial ecology and engineering so that novel approaches to manipulating systems can be developed. For example it has been hypothesised that it may be possible to develop more resilient communities by changing operational conditions, rather than letting a community become specialised [138, 149]. Potential effects that a perturbation may have on a microbial community in AD are illustrated in Figure 1. Filling the gaps of knowledge highlighted on this figure will enable optimisation of the AD process and also contribute to the field of microbial ecology in general.

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