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A Microfluidic Platform for The Simultaneous Quantification of Methanogen Populations in Anaerobic Digestion Processes

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# Summary

Methanogens are a diverse group of archaea that play a critical role in the global carbon cycle. The lack of appropriate molecular tools to simultaneously quantify numerous methanogenic taxa, however, has largely limited our ability to study these communities in a wide variety of habitats, such as anaerobic digesters (ADs). In this study, 34 probe-based quantitative PCR (qPCR) assays were designed to target all known methanogenic genera within the archaeal phylum *Euryarchaeota*. These qPCR assays were adapted to a high-throughput microfluidic platform, which allowed for the simultaneous detection and absolute quantification of numerous taxa in a single run. The resulting microfluidic qPCR (MFQPCR) platform was successfully used to decipher structure–function relationships among methanogenic communities in four laboratory-scale digesters exposed to a transient organic overload. Twelve of the 34 genera targeted in the MFQPCR were detected in the ADs, similar to results obtained using high-throughput sequencing. The MFQPCR platform and conventional qPCR assays also generated similar quantitative results. The MFQPCR tool developed here will help optimize AD technologies for efficient waste treatment and enhanced biogas production and can facilitate studies that will increase our understanding of methanogenic communities in other environments.

# Introduction

Methanogenic archaea, collectively referred to as the methanogens, are strict anaerobes and produce methane gas as an end-product of their metabolism (Schink, **1997**; Thauer *et al*., **2008**). These microbes can utilize substrates derived from the breakdown of complex organic matter (e.g. carbon dioxide, acetate) in anaerobic environments that are low in external electron acceptors (Schink, **1997**). These environments include freshwater and marine sediments, rice-field soils, human and animal gastrointestinal tracts, landfills and wetlands (Liu and Whitman, **2008**).

Methanogens play an indispensable role in anaerobic digestion processes. This technology is widely used for the treatment of municipal and industrial wastes, as well as in the recovery of methane that can be used as a source of renewable energy. Methanogens are sensitive to changes in environmental conditions, such as pH, as well as concentrations of volatile fatty acids (VFAs) and ammonia (Chen *et al*., **2008**). In most cases, these changes are a result of fluctuations in operational parameters, such as organic loading rate, waste composition, toxicant concentrations, hydraulic retention time and temperature (Karakashev *et al*., **2005**; Hori *et al*., **2006**; Demirel and Scherer, **2008**; Krakat *et al*., **2010**; Franke-Whittle *et al*., **2014**). Moreover, the microbial community structure in anaerobic digesters (ADs) can vary greatly and are associated with differences in functional performance (Tale *et al*., **2011**; Bocher *et al*., **2015**; Morris *et al*., **2016**; Venkiteshwaran *et al*., **2017**). Therefore, an understanding of methanogen population dynamics is essential to aid in the prediction and effective control of AD process operations (Carballa *et al*., **2015**).

The advent of high-throughput DNA sequencing (HTS) technology has provided insight into structure–function relationships in ADs. However, one limitation of this technology is that the data generated are semiquantitative, or relative, and that the observed dynamics may not accurately reflect densities of specific taxa. This results in less accurate and sometimes erroneous interpretations (Props *et al*., **2017**). For example, results from HTS analyses may point to an enrichment of taxa or an increase in their relative abundances, but this may not correlate with an increase in absolute abundance or vice versa. This could be a major issue in AD monitoring studies, because HTS would not be able to detect changes in minor populations due to the presence of dominant taxa in the community. The limitations are particularly onerous in the monitoring methanogens, because this functionally critical guild is present in lower relative abundance (<10%) compared to bacteria (Sundberg *et al*., **2013**; Luo *et al*., **2016**; Mei *et al*., **2017**). Consequently, a comprehensive understanding of methanogen populations in AD is currently limited due to lack of appropriate quantification methods. Accurate quantitative data are needed to generate robust quantitative structure–activity relationships (Venkiteshwaran *et al*., **2017**) and to improve the predictability of current mathematical models such as ADM1 (Batstone *et al*., **2002**).

One promising approach to overcome these quantification issues is by the use of a microfluidic quantitative PCR (MFQPCR) platform, in which multiple quantitative PCR (qPCR) assays are run simultaneously in nanoliter-volume chambers present on a chip. The qPCR reagents and DNA samples within the MFQPCR array are mixed automatically by an integrated fluidic circuit controller, which can dramatically reduce the amount of time, labour, and reagents required, compared with conventional qPCR systems. This platform has been successfully applied in different environmental matrices to detect water and food-borne pathogens (Ishii *et al*., **2013**; Ishii, Nakamura, *et al*., **2014**; Zhang and Ishii, **2018**), viruses (Ishii, Kitamura, *et al*., **2014**), antibiotic resistance and heavy metal resistance genes (Sandberg *et al*., **2018**) and nitrogen cycling genes (Oshiki *et al*., **2018**).

Prior to adaptation to the MFQPCR platform, however, many taxon-specific qPCR assays must be developed for accurate and comprehensive coverage of the methanogen community. Currently, the majority of currently available, genus specific, qPCR assays for methanogens are either not specific enough or have limited coverage (reviewed in the study by Narihiro and Sekiguchi, **2015**; **2011** and summarized in the Supporting Information Table **S1**). Notably, qPCR assays are not available for several archaeal genera including some that are frequently detected in ADs, such as *Methanococcoides*, *Methanolobus*, *Methanomassiliicoccus* and *Methanoregula*.

In this study, a microfluidic qPCR platform was developed for the simultaneous detection and high-throughput quantification of methanogens, specifically those involved in AD processes. The objectives of this study were to (i) design and validate 34 probe-based qPCR assays to target all known methanogenic genera; (ii) adapt the newly developed qPCR assays to a microfluidics platform; and (iii) use the microfluidic platform to decipher structure–function relationships among methanogenic communities in laboratory-scale ADs exposed to an organic material overload.

# Results and discussion

## Assay design and validation

### *In silico validation*

In this study, 34 novel genus-specific qPCR primer sets were designed for the quantification of methanogens within the phylum *Euryarchaeota* (Table **1**). The newly designed primers and probes were robust and highly specific, and their PCR products size ranged from 117 to 472 bp (Table **1**). Although several qPCR primers and probes are currently available to quantify methanogens (summarized in the Supporting Information Table **S1**), none exist to detect 17 of these genera, 11 of which have been previously detected in ADs.

**Table 1.**Characteristics of primer and probe sets designed in the study.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Assay No.** | **Genus targeted** | **Primer/Probe** | **Sequence (5′–3′)** | **Coverage (%)** | **Product (bp)** |
| 1 | *Methanobacterium* | Mbact-1013F | AYGAYCTTGCTTGACAAGCT | 96.4 | 348 |
|  |  | Mbact-1138P | ATGCCGGGCACACTAAGGGGACCG | 95.0 |  |
|  |  | Mbact-1341R | ATGACACGCGATTACTACGC | 95.0 |  |
| 2 | *Methanobrevibacter* | Mbrev-208F | TTTTCGCCTAAGGATGGGTC | 83.4 | 208 |
|  |  | Mbrev-363P | AACCTCCGCAATGTGAGMAATCGC | 84.5 |  |
|  |  | Mbrev-402R | ATCCCGTTAAGAATGGCACT | 87.2 |  |
| 3 | *Methanocalculus* | Mcalc-670F | GACGTACTTCGGGGGTAGGA | 96.9 | 328 |
|  |  | Mcalc-701P | TAATCCCCGAGGGACGACCGATGG | 90.6 |  |
|  |  | Mcalc-973R | TCGTATCCGGTGAGTTGTCC | 89.1 |  |
| 4 | *Methanocaldococcus* | Mcald-464F | AAACAGCTCCGGGAATAAGG | 97.1 | 348 |
|  |  | Mcald-691P | TGAAATGCGTTGATCCCTGGGGGA | 97.1 |  |
|  |  | Mcald-808R | TAGTCCGCAGAGTTTACAGC | 97.1 |  |
| 5 | *Methanococcoides* | Mcoid-444F | TGTGTAAACGGCATGTGWTA | 90.9 | 183 |
|  |  | Mcoid-582P | AGCCGGTTTGATCAGTTCTTCGGG | 92.2 |  |
|  |  | Mcoid-636R | CCCAAGTCTGACAGTATTCC | 94.8 |  |
| 6 | *Methanococcus* | Mcocc-232F | ACTGCGCTCGATTAGGTAGT | 85.2 | 472 |
|  |  | Mcocc-652P | TGGGACCGGGAGAGGACAAGGGTA | 83.3 |  |
|  |  | Mcocc-726R | CGTTCCAGACAAGTGCCTTC | 85.2 |  |
| 7 | *Methanocorpusculum* | Mcorp-656F | ACTGGGAGAGGTAAACCGTA | 97.7 | 229 |
|  |  | Mcorp-710P | AGGGACGACCTATGGCGAAGGCAG | 96.3 |  |
|  |  | Mcorp-861R | TGGTGCGTTTCACGATTTCT | 98.2 |  |
| 8 | *Methanoculleus* | Mcull-570F | TAAAGCGTTCGTAGCTGGGT | 96.5 | 455 |
|  |  | Mcull-702P | AATCCTCGAGGGACCACCTGTGGC | 95.0 |  |
|  |  | Mcull-1000R | CTTCAGCCCGGCAATCATTC | 95.8 |  |
| 9 | *Methanofollis* | Mfoll-677F | TCTGGGGGTAGGAGTGAAAT | 85.7 | 332 |
|  |  | Mfoll-728P | AGGCGTCTCACCAGAACGACTCCG | 85.7 |  |
|  |  | Mfoll-984R | TCATATCGCTGTCCTACCCG | 90.5 |  |
| 10 | *Methanogenium* | Mgenm-273F | AGCCTGTAATCGATACGGGT | 90.7 | 363 |
|  |  | Mgenm-407P | CCGTATACGCGGGCTGTCCAGGTG | 90.7 |  |
|  |  | Mgenm-661R | CGGAGTACCTCTCACCTCTC | 95.3 |  |
| 11 | *Methanohalobium* | Mhlbm-586F | GGTTTGATCAGTCCATTGGG | 100.0 | 257 |
|  |  | Mhlbm-702P | AATCCCTGTAGGACCACCAGTTGC | 100.0 |  |
|  |  | Mhlbm-826R | CGTACCATCCCTAACACCTA | 100.0 |  |
| 12 | *Methanohalophilus* | Mhlps-134F | CCCTTGGGTTCAGCATAACC | 96.5 | 288 |
|  |  | Mhlps-358P | CGCGAAACCTTTACACTGCGGGAA | 100.0 |  |
|  |  | Mhlps-399R | CATTGTATGCTGGCACTCGG | 96.5 |  |
| 13 | *Methanolacinia* | Mlaci-442F | GGTGTGYAAAAAGCATCTGA | 100.0 | 275 |
|  |  | Mlaci-622P | CCGGCGGGCGTCCAAGAGATACTG | 100.0 |  |
|  |  | Mlaci-727R | CCGTCCTGGTAAGATGCCTT | 100.0 |  |
| 14 | *Methanolinea* | Mlnea-398F | CCCGAGTGCCCGTAAATTCG | 86.9 | 308 |
|  |  | Mlnea-562P | ACTGGGCTTAAAGCGTCCGTAGCT | 77.0 |  |
|  |  | Mlnea-731R | GGAGCCGTTCTAGTGAGACG | 73.8 |  |
| 15 | *Methanolobus* | Mlobs-587F | GTTYGATCAGTCTTCCGGGA | 89.5 | 413 |
|  |  | Mlobs-815P | ACGATGCTCGCTAGGTGTCTGGGA | 90.5 |  |
|  |  | Mlobs-974R | GTCGCCCCAGGTGAGTTTTC | 88.4 |  |
| 16 | *Methanomassiliicoccus* | Mmsls-851F | AGTGYCGGAGAGAAGTTGTT | 89.1 | 230 |
|  |  | Mmsls-934P | GCAACGGGWGGAGCGTGCGGTTYA | 89.9 |  |
|  |  | Mmsls-1067R | GAACGCCYYACGGTACGAAC | 88.9 |  |
| 17 | *Methanomethylovorans* | Mmtyl-596F | GTCTTCCGGGAAATCTGACG | 95.1 | 463 |
|  |  | Mmtyl-669P | AGAGGTACTACGGGGGTAGGAGTG | 100.0 |  |
|  |  | Mmtyl-1020R | TCCTCTCAGCGATTCTGGTA | 95.1 |  |
| 18 | *Methanomicrobium* | Mmcbm-622F | CTGTGAGGCGTCTAAGAGAT | 96.2 | 263 |
|  |  | Mmcbm-832P | TGCCTGTAACTACGAGTTACCGGG | 98.1 |  |
|  |  | Mmcbm-859R | GCACGTTTCACGTTTTCACT | 96.2 |  |
| 19 | *Methanimicrococcus* | Mmicr-1057F | GTCGTCGCCAGTTCGTACTG | 94.1 | 262 |
|  |  | Mmicr-1179P | AGGTGCGGGCTACGGTAGATCAGT | 98.0 |  |
|  |  | Mmicr-1290R | TCGATCCGAACTTCGAACGG | 96.1 |  |
| 20 | *Methanoplanus* | Mplns-191F | TTCTTTRAYCGAAAGTTCCG | 89.5 | 347 |
|  |  | Mplns-395P | AACCCCGAGTGCCCGTATAYGCGG | 94.7 |  |
|  |  | Mplns-570R | KTCMAGCTACGAACGCTTTA | 94.7 |  |
| 21 | *Methanosalsum* | Msalm-587F | GTTTGATCAGTCCTCTGGGA | 100.0 | 255 |
|  |  | Msalm-630P | CTKCCAGGGGATACTGTCAGACTT | 100.0 |  |
|  |  | Msalm-821R | CATCCCTGACACATAGCGAG | 100.0 |  |
| 22 | *Methanosarcina* | Msarc-542F | CCCGAGTGGTGATCGTKATT | 91.1 | 300 |
|  |  | Msarc-581P | TAGCCGGTTTGGTCAGTCCTCCGG | 93.3 |  |
|  |  | Msarc-821R | CATGCCTGACACCTAGCGAG | 94.1 |  |
| 23 | *Methanosphaera* | Msphr-491F | TAGAATAAGAGCTGGGCAAG | 92.9 | 275 |
|  |  | Msphr-658P | CGGGAGAGGTTAGAGGTACTACCG | 93.4 |  |
|  |  | Msphr-744R | GTTACTCACCGTCAAGATCG | 92.4 |  |
| 24 | *Methanospirillum* | Msprl-383F | CCGTGATAAGGAAACCCCGA | 94.9 | 285 |
|  |  | Msprl-562P | ACTGGGCTTAAAGGGTCCGTAGCT | 93.8 |  |
|  |  | Msprl-693R | CCACCGGGATTACAGGATTT | 94.9 |  |
| 25 | *Methanothermobacter* | Mtbtr-1119F | CTTAGTTACCAGCGGRACCC | 93.6 | 186 |
|  |  | Mtbtr-1229P | CGGGCTACAATGGCCTGGACAATG | 97.4 |  |
|  |  | Mtbtr-1275R | ACCTGGTTTAGGGGATTACC | 97.4 |  |
| 26 | *Methanothermococcus* | Mtmcs-704F | TCCCTGGAGGACCACCTATG | 95.0 | 436 |
|  |  | Mtmcs-980P | TCACCAGGGGCGACAGCATGATGA | 90.0 |  |
|  |  | Mtmcs-1122R | CGGAGGAAGAAGTSGCAACA | 95.0 |  |
| 27 | *Methanothermus* | Mtrms-54F | ATGCAAGTCGAACGGGCCTT | 100.0 | 148 |
|  |  | Mtrms-171P | ATCCCGGATAGGCGAGGTCTCCTG | 83.3 |  |
|  |  | Mtrms-193R | AAAAGACCTTTCGGCGAGGC | 100.0 |  |
| 28 | *Methanothrix* | Mthrx-659F | GGGAGAGGTGAGAGGTACYT | 93.0 | 393 |
|  |  | Mthrx-751P | GACGGYAAGGGACGAAAGCTAGGG | 92.9 |  |
|  |  | Mthrx-1028R | GCACCACCTCTCAGCKAATC | 92.9 |  |
| 29 | *Methanotorris* | Mtorr-587F | GCCCAGTAAGTCCCTGCTTA | 100.0 | 122 |
|  |  | Mtorr-645P | CTGGGCTTGGGACCGGGAGAGGGC | 91.7 |  |
|  |  | Mtorr-689R | GGGGATCAACACATTTCACC | 100.0 |  |
| 30 | *Methermicoccus* | Mtrcs-64F | TATGYTCYCCTTCGGGRGRG | 75.0 | 117 |
|  |  | Mtrcs-133P | ACCCTTGGGACRGGSATAACCCCG | 100.0 |  |
|  |  | Mtrcs-177R | TCCAGCCTCCATGGTCTATC | 75.0 |  |
| 31 | *Methanocella* | Mcell-604F | GGAAATCCAGCGGCTTAACC | 92.0 | 400 |
|  |  | Mcell-643P | TACATTGCTTGGGACTGGGAGAGG | 90.0 |  |
|  |  | Mcell-979R | CAACTGTCGATCCCGGTAAG | 100.0 |  |
| 32 | *Methanopyrus* | Mpyrs-239F | CCGATTAGGTAGTTGGCGGG | 100.0 | 384 |
|  |  | Mpyrs-275P | CCGATAATCGGTACGGGCGGTGAG | 100.0 |  |
|  |  | Mpyrs-638R | GTCCCAAGTCCCGCAGTTTC | 100.0 |  |
| 33 | *Methanoregula* | Mregu-1120F | ACAGTTGCYAGCTCGTCCYC | 92.9 | 275 |
|  |  | Mregu-1239P | TGGGTGGGACAATGGGTATCGACA | 98.4 |  |
|  |  | Mregu-1367R | AGCAGGGACATATTCACCGT | 92.9 |  |
| 34 | *Methanosphaerula* | Msphl-209F | ATCGCCTTAGGATGGGTCTG | 100.0 | 374 |
|  |  | Msphl-414P | TAAACGCGGGCTGTCCATCTGCTT | 100.0 |  |
|  |  | Msphl-616R | TAAACGCCTGTCGGTTAAGC | 100.0 |  |

F = forward primer, P = probe, R = reverse primer; W = A/T, Y = C/T, R = A/G, S = G/C, K = G/T and M = A/C; RDP Release 11, Update 5

*In silico* analysis using Probe Match function of the Ribosomal Database Project (RDP) revealed that all qPCR assays cover ≥83% of the target sequences except for two assays specific to *Methanolinea* (74%) and *Methermicoccus* (75%). Although individual primers and probes sometimes matched with non-target sequences, no single non-target sequences were detected when primers and probe were used in combination, suggesting that the qPCR assays developed for this work were highly specific to target methanogens. All of the assays developed in this study displayed comparable or greater coverage and specificity than the genus-specific primers and probes that were previously designed (summarized in the Supporting Information Table **S1**).

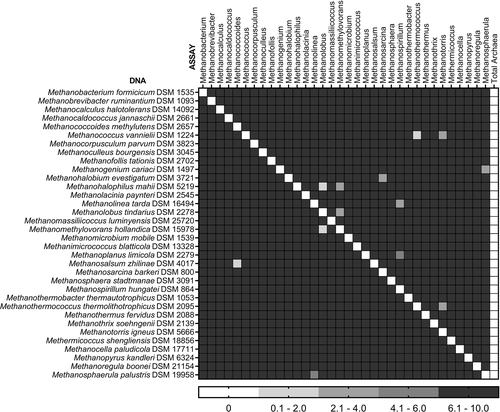
### *Standard curves*

Standard curves constructed from a series of 10-fold plasmid DNA dilutions displayed a linear dynamic range spanning six orders of magnitude (from 108 to 102 copies; Supporting Information **Table S2**). The limit of detection (LOD) using conventional qPCR was at least 100 copies per reaction. Amplification efficiencies ranged from 89.4 to 107%, with the exceptions of *Methanopyrus* (59.6%) and *Methanotorris* (74.1%). Both of these genera are extremophiles and have high GC content, which might have influenced the low amplification efficiencies (Burggraf *et al*., **1990**; Kurr *et al*., **1991**). These groups have not been reported in ADs; therefore, poor performance of these two assays does not negatively influence our main objective. The regression coefficient (*R*2) for all standard curves was above 0.995 and large. Ct values (≥32) were observed for no-template controls.

Because all of the qPCR assays were performed under identical thermal conditions, they were considered appropriate for simultaneous quantification on a single microfluidics chip. When using the MFQPCR platform, the amplification efficiencies of most assays (27/34) ranged from 86.2% to 108%, with the exceptions of *Methanocaldococcus* (57.9%), *Methanococcus* (59.3%), *Methanoculleus* (71.5%), *Methanomethylovorans* (80.0%), *Methanotorris* (62.5%), *Methanocella* (70.3%) and *Methanopyrus* (which failed) (Supporting Information **Table S2**). The MFQPCR platform was less sensitive than conventional qPCR and had a higher LOD of 1000 gene copies per reaction. This was likely due to the small sample volume (6.7 nL) used in the MFQPCR platform. The LOD could be lowered further by performing specific target amplification (STA) prior to MFQPCR. The STA reaction is a multiplex PCR, which employs primers used for the MFQPCR with a smaller number of PCR cycles. This preamplification increases the number of copies to a detectable level and without significant bias as was previously demonstrated by others (Ishii *et al*., **2013**; Ishii, Kitamura, *et al*., **2014**).

### *Experimental validation*

To determine whether there was non-specific amplification, MFQPCR was performed using all 34 assays and 34 methanogen DNA extracts. All 34 qPCR assays specifically amplified the target methanogens, while non-target amplification was observed in only 1% (13/1122) of the comparisons (=34 assays × 33 non-target samples; Fig. **1**). The Ct values for the non-target samples that showed amplification were two to five cycles larger than that for the target. These non-target amplification products were also observed when using conventional qPCR (Supporting Information **Table S3**). The cross-amplification could be attributable to phylogenetic relatedness between the target and non-targets as most of the non-targets that were amplified belonged to the same family as the target genus. Regardless, the non-target amplification found in some assays will not influence this study's aim of conducting population dynamic studies in ADs. Most of these assays target methanogens that have not been found in ADs, most likely due to their specific physiological growth requirements (e.g. halophilic methylotrophic methanogens). Nonetheless, if necessary, it has been suggested that another method be used to differentiate the quantitative contribution of methanogen groups that have been previously detected in ADs and that showed up as a non-target (e.g. *Methanolinea*).

[](https://ami-journals.onlinelibrary.wiley.com/cms/asset/066d2f69-afc3-4bac-9e73-ff2d5442549b/emi14589-fig-0001-m.jpg)

**Figure 1** Specificity of qPCR assays designed in this study. Specific amplifications were verified experimentally using DNA from 34 archaeal cultures. These results were obtained using microfluidic qPCR and confirmed with conventional qPCR. Ct values above the detection limit for each assay were considered as positive. Legend shows Ct difference between target and non-target DNA

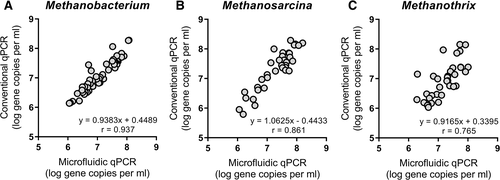
## Application of MFQPCR to monitor methanogenic communities in anaerobic digesters

### *Efficacy and reliability*

The MFQPCR platform developed in this study was successfully used to quantify methanogenic genera in four laboratory-scale ADs at 13 time points per digester. Among the 34 assays, 12 genera were detected within quantifiable ranges in these ADs: *Methanobacterium*, *Methanothrix*, *Methanosarcina*, *Methanomassiliicoccus*, *Methanoculleus*, *Methanolinea*, *Methanomethylovorans*, *Methanospirillum*, *Methanofollis*, *Methanoregula*, *Methanobrevibacter* and *Methanolobus* (Supporting Information **Table S4**). These genera have been previously reported to be present in a wide variety of full-scale ADs (Leclerc *et al*., **2004**; Sundberg *et al*., **2013**; Luo *et al*., **2016**).

High-throughput DNA sequencing confirmed that the MFQPCR platform was successful in detecting all known methanogenic genera in our samples (Supporting Information **Table S4**). In addition, a chi-square test showed that the relative proportion of the top three most abundant genera were not significantly different between these two methods: *Methanobacterium* (*p* = 0.472), *Methanosarcina* (*p* = 1.000) and *Methanothrix* (*p* = 0.790). These genera were also the top three most frequently detected in our samples and constituted 86.8% of the total methanogen relative abundance (Supporting Information **Table S4**).

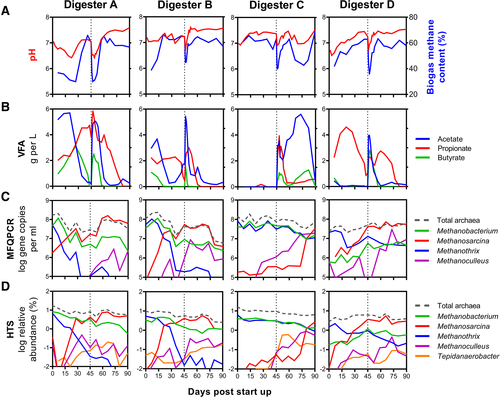
Conventional qPCRs were also performed on these three genera to verify the results obtained by MFQPCR (Fig. **2**). Highly significant correlations were observed between the two methods: *Methanobacterium* (*r* = 0.934; *p* < 0.0001), *Methanosarcina* (*r* = 0.916; *p* < 0.0001) and *Methanothrix* (*r* = 0.725; *p* < 0.0001), suggesting that the quantitative data obtained by MFQPCR were as accurate as those obtained using conventional qPCR.

[](https://ami-journals.onlinelibrary.wiley.com/cms/asset/d0bc0ba3-4fc8-43f6-8b2b-6a3fa3da2b5f/emi14589-fig-0002-m.jpg)

**Figure 2** Correlation between concentrations measured by microfluidic and conventional qPCR. Quantitative data for the three most abundant genera (a) *Methanobacterium*, (b) *Methanosarcina*, and (c) *Methanothrix*, in samples from four laboratory-scale ADs were used for comparison. The linear regression equation and Spearman's rank correlation coefficient are also shown.

### *Dynamics of methanogen populations involved in acetate utilization*

Quantitative results obtained from the MFQPCR were used to examine structure–function relationships among methanogenic communities in four laboratory-scale ADs exposed to an organic material overload. Each digester was seeded with different inocula and displayed different physicochemical profiles during the entire operational period (Fig. **3**a and b).

[](https://ami-journals.onlinelibrary.wiley.com/cms/asset/19566e6c-3195-45ed-b9ae-33a937a18c0c/emi14589-fig-0003-m.jpg)

**Figure 3** Microbial structure–function analysis of four laboratory-scale ADs exposed to an overload perturbation. (a) pH and biogas methane content. (b) VFA concentrations. (c) Microfluidic qPCR. (d) HTS. All digesters were operated at an OLR of 2 g COD LR−1 day−1, except on day 45 when it was overloaded with 10× feed.

In digesters A, B and D, *Methanosarcina* replaced *Methanothrix* as the dominant acetoclastic methanogen, and all three underwent a start-up phase characterized by high VFA concentrations and low pH values. In these digesters, an observed increase in acetate utilization between days 20 and 40 was associated with an increase in the population of *Methanosarcina*. In contrast, digester C had much faster start-up times (less than a week) with no detectable VFAs, a neutral pH and higher biogas methane content. Digester C was seeded with inoculum from a bench-scale digester fed the same feed as employed in this study, and C exhibited stable populations of *Methanothrix*, which was the dominant acetate-utilizing methanogen in this digester, throughout the study period.

Organic overloading on day 45 with feed at a 10-fold greater organic strength resulted in a dramatic increase in VFA concentrations in the digesters (Fig. **3**b). In all digesters, the pH and biogas methane content dropped to the lowest concentrations observed throughout the study within 2 days of the overload (Fig. **3**a). All other functional parameters (pH, acetate, butyrate and methane content) returned to pre-perturbation levels within 10 days of the overload in digesters A, B and D, although the propionate concentration remained relatively high. Digesters A, B and D, with a greater abundance of *Methanosarcina*, were better able to tolerate the elevated acetate concentrations that resulted from the overload.

In contrast, the *Methanothrix*-dominated digester C became functionally unstable after the perturbation. This was characterized by VFA buildup between 52 and 73 days, which resulted in a drastic decline in pH and biogas methane content. Digester C stabilized only after the emergence of *Methanosarcina*, which increased in abundance (>two orders of magnitude between 73 and 80 days) after high acetate concentrations appeared. Interestingly, the rapid growth of *Methanosarcina* in this digester was observed only after acetate levels increased to 3 g per L, which is considered the maximum acetate tolerance limit for *Methanothrix* (De Vrieze *et al*., **2012**). In general, *Methanosarcina* offers several physiological features not found in *Methanothrix* that are beneficial during high-VFA concentration, stressed conditions (De Vrieze *et al*., **2012**).

Drastic increases in the abundance of *Methanoculleus,* a hydrogenotrophic methanogen, was observed digesters A (59–80 d), B (52–59 d), C (45–80 d) and D (59–73 d) in response to elevated acetate concentrations (Fig. **3**c). The increase in *Methanoculleus* abundance also corresponded with substantial increases in the relative abundance of *Tepidanaerobacter* (Fig. **3**d). Some species within this genus, such as *Tepidanaerobacter acetatoxydans,* have been reported to oxidize acetate in syntrophic association with *Methanoculleus* spp. (Westerholm *et al*., **2011**). It should be noted that no dramatic increase in *Tepidanaerobacter* was observed in digesters A, B and D, which had high abundances of *Methanosarcina* spp. before overload (>107 copies per ml, Fig. **3**c). This suggests that syntrophic acetate oxidation is triggered if the digester does not have a sufficient population of *Methanosarcina* to rapidly utilize acetate during periods of its increased production. In contrast, *Methanoculleus* responded before *Methanosarcina* in digester C, and this was correlated with an increased abundance of *Tepidanaerobacter*. Unfavourable conditions (such as high VFA and low pH) most likely inhibited the growth of *Methanosarcina,* as well as its ability to respond to increased acetate concentrations. Nonetheless, *Methanosarcina* greatly increased in abundance after more favourable growth conditions were established (such as an increased pH) in digester C between 80 and 90 days.

# Conclusions

In this study, we developed a sensitive and specific method for the rapid, high throughput, and simultaneous quantification of 34 methanogenic genera. The MFQPCR technique overcomes many inherent limitations of current molecular methods, such as high-throughput DNA sequencing and singleplex qPCRs by allowing simultaneous and absolute quantification of numerous taxa in a single run. The MFQPCR platform was successfully used to assess methanogen population dynamics in ADs, and its application to laboratory-scale ADs exposed to an organic overload perturbation identified several methanogens that were critical for AD function. Future studies should also include assays targeting other key functional taxa, such as syntrophic VFA degraders. These new tools will help optimize digester technologies for efficient waste treatment and enhanced biogas production, as well as provide an increase in our understanding of methanogenic communities in other environments.

# Experimental procedures

## Anaerobic digester setup and operation

Four laboratory-scale ADs were established in 160 ml serum bottles and incubated on a shaker table (at 100 rpm) at 37 ± 1°C for 90 days. Different inocula were used in an effort to obtain different starting microbial communities. Three laboratory-scale ADs were seeded with biomass from different full-scale ADs treating specific waste: digester A (food and beverage waste), digester B (ethanol waste) and digester D (yogurt waste). The fourth digester (C) was seeded with biomass from a bench-scale digester fed non-fat dry milk (Roundy's; Milwaukee, WI, USA). All ADs contained basal nutrient medium (Speece, **2008**) were sparged with a N2:CO2 gas mixture (7:3 ratio v/v) and fed synthetic industrial wastewater composed of non-fat dry milk containing 52% w/w sugars and 35% w/w proteins. The organic loading rate was 2 g COD LR−1 day−1, except on day 45, when the ADs were shock overloaded with feed at 10 times greater organic strength (20 g COD LR−1 day−1). Each day, 4 ml of effluent was discarded and replaced with 4 ml of feed to maintain a 15-day hydraulic retention time.

## Analytical methods

Effluent samples (1 ml) for VFA analysis were centrifuged at 10 000 × *g* for 10 min. Supernatant was filtered through a 0.45 μm pore syringe filter (Bonna-Agela Technologies, Wilmington, DE, USA) and immediately acidified with 1% (v/v) phosphoric acid. VFA concentrations were measured using a gas chromatograph (7890A GC system; Agilent Technologies, Irving, TX, USA) equipped with a flame ionization detector as described previously (Venkiteshwaran *et al*., **2016**). Biogas production was measured by inserting a needle with a wetted glass-barrel syringe through serum bottle septa and noting the displacement of the wetted barrel. The methane content of the biogas was measured using a gas chromatograph equipped with a thermal conductivity detector (Venkiteshwaran *et al*., **2016**). Effluent pH was measured using a bench-top pH meter (Orion; Thermo Fisher Scientific Waltham, MA, USA) by standard methods (Clesceri *et al*., **1998**).

## DNA extraction

Effluent samples (1 ml) were collected for molecular analyses from each digester on days 0, 7, 14, 21, 28, 35, 45, 52, 59, 66, 73, 80 and 90 and centrifuged at 10 000 × *g* for 10 min. DNA was extracted from the pellets using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA). DNA was quantified using a Nanodrop (ND-1000; ThermoScientific, Waltham, MA, USA). Extracted DNA was stored in 10 mM Tris buffer (pH 8) at −80°C until subsequent analysis.

## Primers and probes

In this study, 34 novel primer/probe sets were designed to target all known genera with at least one cultured representative of methanogens. All of them belong to the phylum *Euryarchaeota*. For each genus of interest, all available 16S rRNA gene sequences (≥ 1200 bp) were retrieved from the Hierarchy Browser in the Ribosomal Database Project-Release 11, Update 5 (Cole *et al*., **2014**) and aligned using MUSCLE (Edgar, **2004**). The resulting consensus sequences were examined for the suitability as genus-specific primer/probes using the Probe Match function at RDP (**https://rdp.cme.msu.edu/probematch/search.jsp**). Degenerate bases were introduced, when necessary, to increase target coverage. Sequences were selected if they satisfied the following criteria: (i) ≥ 80% coverage of target group, (ii) ≤ 10% non-target hits and (iii) at least two mismatches between closely related taxa. Primer characteristics such as GC content, melting temperature (*T*m) and their potential to form secondary structures were evaluated using the OligoAnalyzer version 3.1 software (Integrated DNA Technologies, Coralville, IA, USA). The *T*m of all probes was designed to be between 5 and 10°C higher than the *T*m of the forward and reverse primers in corresponding sets. All primers and probes were synthesized by Integrated DNA Technologies. Probes were labelled with 6-FAM at their 5′ end, the Iowa Black fluorescent quencher at their 3′ end and an internal ZEN quencher that was inserted between the 9th and 10th base from the 5′ end of the probe sequence.

## DNA standards for qPCR

DNA standards for each genus were constructed using near full-length 16S rRNA gene-amplicons produced by PCR, with modified versions of universal primers 8F and 1492R from DNA obtained from 34 cultures. Thirty-two strains were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) (Braunschweig, Germany), and cultures of *Methanoregula boonei* and *Methanosphaerula palustris* were generously provided by Dr. Suzanna Brauer (Appalachian State University) and Dr. Hinsby Cadillo-Quiroz (Arizona State University) respectively (Table **2**). The PCR products were cloned into pCRTM 2.1-TOPOTM vector (Invitrogen, Carlsbad, CA, USA) and transformed into competent *Escherichia coli* DH5 cells via chemical transformation, as per the manufacturer's recommendation. Positive transformants were grown overnight at 37°C in LB broth with ampicillin (100 μg per ml). Plasmids were purified with a QIAprep Spin MiniPrep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified using the Qubit 1.0 fluorometer (Invitrogen). All plasmids were sequenced to confirm the presence of the correct insert (Supporting Information **Table S5**). Plasmid DNA was normalized to 1010 copies per μL and diluted 10-fold to obtain a dilution series ranging from 109 to 102 copies per μL. Dilution series were used to make standard curves to determine the linear dynamic range for each qPCR assay developed in this study. For MFQPCR, all 34 standards were pooled and diluted to make a serial dilution of standard mixture with concentrations ranging from 108 to 102 gene copies per μL.

**Table 2.**List of archaeal type strains used in this study

|  |  |  |
| --- | --- | --- |
| **No.** | **Type species** | **DSM No.** |
| 1 | *Methanobacterium formicicum* | 1535 |
| 2 | *Methanobrevibacter ruminantium* | 1093 |
| 3 | *Methanocalculus halotolerans* | 14 092 |
| 4 | *Methanocaldococcus jannaschii* | 2661 |
| 5 | *Methanococcoides methylutens* | 2657 |
| 6 | *Methanococcus vannielii* | 1224 |
| 7 | *Methanocorpusculum parvum* | 3823 |
| 8 | *Methanoculleus bourgensis* | 3045 |
| 9 | *Methanofollis tationis* | 2702 |
| 10 | *Methanogenium cariaci* | 1497 |
| 11 | *Methanohalobium evestigatum* | 3721 |
| 12 | *Methanohalophilus mahii* | 5219 |
| 13 | *Methanolacinia paynteri* | 2545 |
| 14 | *Methanolinea tarda* | 16 494 |
| 15 | *Methanolobus tindarius* | 2278 |
| 16 | *Methanomassiliicoccus luminyensis* | 25 720 |
| 17 | *Methanomethylovorans hollandica* | 15 978 |
| 18 | *Methanomicrobium mobile* | 1539 |
| 19 | *Methanimicrococcus blatticola* | 13 328 |
| 20 | *Methanoplanus limicola* | 2279 |
| 21 | *Methanosalsum zhilinae* | 4017 |
| 22 | *Methanosarcina barkeri* | 800 |
| 23 | *Methanosphaera stadtmanae* | 3091 |
| 24 | *Methanospirillum hungatei* | 864 |
| 25 | *Methanothermobacter thermautotrophicus* | 1053 |
| 26 | *Methanothermococcus thermolithotrophicus* | 2095 |
| 27 | *Methanothermus fervidus* | 2088 |
| 28 | *Methanothrix soehngenii* | 2139 |
| 29 | *Methanotorris igneus* | 5666 |
| 30 | *Methermicoccus shengliensis* | 18 856 |
| 31 | *Methanocella paludicola* | 17 711 |
| 32 | *Methanopyrus kandleri* | 6324 |
| 33 | *Methanoregula boonei* | 21 154 |
| 34 | *Methanosphaerula palustris* | 19 958 |

## Conventional qPCR

Conventional qPCR was done using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture (10 μl) contained 1× iTaq™ Universal Probes Supermix (Bio-Rad, Hercules, CA, USA), 500 nM each forward and reverse primer, 100 nM probe and 2 μL DNA template. PCR was performed, in duplicate, under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each qPCR run included DNA standards and no-template controls. ROX was used as a passive reference dye.

## Microfluidic qPCR

MFQPCR was performed using either a 48.48 or a 96.96 Dynamic Array chip (Fluidigm, San Francisco, CA, USA) and a BioMark HD System (Fluidigm) as described previously (Ishii *et al*. **2013**). The first run (48.48 chip) was used to determine assay (primer/probe) specificity against the target (*n* = 1) and non-target (*n* = 33) methanogen DNA, and the second run (96.96 chip) was used to quantify methanogens in laboratory-scale ADs. The 10× assay premix (8 μL) for each target (*n* = 35) contained 1× assay loading reagent (Fluidigm), 8 μM Primer Pair Mix and 1 μM probe. The sample premix (8 μL) contained 1× iTaq™ Universal Probes Supermix (Bio-Rad), 1× GE Sample Loading Reagent (Fluidigm) and 3.6 μL of DNA. As the 96.96 chip could only accommodate 96 assays, 24 assays were run in triplicate and 12 assays in duplicate. Aliquots (5 μL) of the assay and sample premixes were loaded into the chip and mixed using an IFC controller MX (for 48.48 chip) or HX (for 96.96 chip) according to the manufacturer's instruction. The chip was then loaded onto BioMark HD System for real-time PCR. PCR was performed under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. To ensure proper mixing of samples and assays in the 96.96 chip, an additional thermal mixing protocol (70°C for 30 min and 25°C for 10 min) was added after 50°C for 2 min step, as per the manufacturer's instructions. Each MFQPCR run included DNA standards and no-template controls. ROX was used as a passive reference dye.

## qPCR data analysis

The threshold cycle (Ct) value was determined using the StepOnePlus™ Software v2.3 (Applied Biosystems) and the Real-Time PCR Analysis software version v.2.1.3 (Fluidigm) for conventional qPCR and MFQPCR respectively. The standard curves were generated by linear regression analysis of the Ct values versus the known concentrations of the DNA standards (log gene copies per μl). The PCR amplification efficiency was calculated from the slope of the regression line as described previously (Bustin *et al*., **2009**). The goodness-of-fit (*R*2) value was also calculated for each standard curve. For environmental samples, the quantity of the target gene was calculated from the Ct values using the standard curves. Values falling below the lowest concentration on the standard curve were treated as negatives.

## High-throughput DNA sequencing

DNA samples (*n* = 52) were sequenced at the University of Minnesota Genomics Centre using universal primers: 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V4 region of the 16S rRNA gene. Sequencing was performed on an Illumina HiSeq platform using a 2 × 250 bp paired-end protocol (Gohl *et al*., **2016**). All fastq files were deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA507417. The sequencing data were processed using RDPipeline (**http://pyro.cme.msu.edu/**; Cole *et al*., **2014**). During the first step, raw paired-end reads were merged, trimmed and low-quality sequences were filtered using Assembler, a modified version of PANDAseq (Masella *et al*., **2012**). Sequence files generated from the initial processing step were checked for chimeras using UCHIME (Edgar *et al*., **2011**). The resultant non-chimeric 16S rRNA gene sequences were assigned to the bacterial and archaeal taxonomy using the RDP Classifier (Wang *et al*., **2007**).

## Statistical analysis

Spearman rank correlation was used to compare quantities obtained by MFQPCR and conventional qPCR, because the Shapiro–Wilk test indicated that the data were not normally distributed. A chi-square test was used to compare the relative abundance of methanogenic genera obtained by MFQPCR and high-throughput DNA sequencing. All statistical analyses were done using XLStat Ecology (Addinsoft, Long Island, NY, USA) and GraphPad Prism v 7.04.

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