A metabolic quotient for methanogenic *Archaea*

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**ABSTRACT**

Biogas production from renewable resources is an alternative to generate energy and concomitantly save fossil fuels and mitigate greenhouse gas emissions. As methanogenesis is a major bottleneck in the biogas process, the determination of the specific activity of methanogenic *Archaea* can be a good indicator of the process state. A new parameter, the metabolic quotient (MQ), was developed to evaluate the specific activity of methanogens. A standard was created from mesophilic maize-fed fermenters to calculate the expected concentration of methanogens for a given methane productivity at stable process stages. The MQ, the ratio of the predicted to the actual concentration of methanogens, defines their metabolic activity. The MQ was able to indicate methanogenic cell stress metabolism and imminent process failure before conventional chemical parameters. As a further approach, the methanogenic activity was determined by quantification of mRNA transcripts in relation to the mcrA/mrtA-gene, coding for a key enzyme subunit of methanogenesis. The cDNA/DNA ratio reflected the specific actual process activity of the methanogens. As both methods are potent parameters for the early detection of process failure, biogas plant operators may avoid economical losses by their preventive application.

**Key words** | mcrA/mrtA, metabolic quotient, methanogenic *Archaea*, qPCR, RT-qPCR

**INTRODUCTION**

According to the German Biogas Association, ca. 7,000 biogas plants were located in Germany in the end of the year 2011. The complex anaerobic digestion of organic substrate is accomplished by the cooperation of different microorganisms. Methanogenesis is often referred to as the bottleneck of the biogas process. This step is performed by methanogenic *Archaea* which cleave acetate or convert hydrogen and carbon dioxide to methane. The key enzyme of methanogenesis is Methyl-CoM-Reductase (Mcr). The gene subunit mcrA/mrtA can be used to infer phylogeny, as the topology of mcrA/mrtA phylogenetic trees is almost identical to that of ribosomal DNA (Luton et al. 2002). The detection of functional genes enables analysis of specifically microbial guilds with a distinct metabolic function.

Several studies using molecular biological methods to analyse the biocenoses of anaerobic digesters have been published (e.g. Yu et al. 2005; Demirel & Scherer 2008; Klocke et al. 2008; Steinberg & Regan 2009). These analyses help to shed light on the microbiological ‘black box’ of fermenters, to obtain more information on the complex microbial process of anaerobic digestion without employing difficult and time consuming cultivation techniques. Besides the identification of methanogenic populations, their quantification is an important task to reveal population fluctuations. Recent progress has been made in the recent years (Yu et al. 2005; Bauer et al. 2008; Steinberg & Regan 2009; Munk et al. 2010), and the detection and quantification of gene expression in environmental microbiology have been improved (Sharkey et al. 2004; Talbot et al. 2008; Freitag & Prosser 2009). Transcription analysis enables exclusive detection of short-lived mRNA produced by active organisms without the potential bias of DNA detection from dormant or dead cells. The transcription of mcrA/mrtA by methanogens is a prerequisite for the production of methane.

The aim of this study was to determine the specific methanogenic activity and its response to environmental changes in biogas fermenters in order to develop an early warning system of microbial stress and process disturbance. In a first step, the concentration of mcrA/mrtA genes in mesophilic, maize-fed fermenters has been determined at various organic loading rates (OLR), and a standard for
stable and efficient process states was created to calculate predicted ‘standard’ concentrations of methanogenic Archaea at given methane productivity rates (Munk et al. 2010). The predicted concentration of methanogens compared with the actual one was defined as metabolic quotient (MQ). In this study we report how this can give information on the actual metabolic activity of the methanogens and their physiological state.

A further goal was to evaluate a second parameter of specific microbial activity which does not rely on gas production data. In this approach, the methanogenic activity was determined by quantification of mcrA/mrtA-transcripts in relation to the respective gene concentration, informing about the actual metabolic transcriptional activity of the methanogenic cells. This study reports, for the first time, the determination of the microorganism-specific methanogenic activity based on a functional gene in biogas plants fed with energy crops.

**METHODS**

**Fermenter management and sampling**

In order to develop the MQ, six mesophilic fermenters fed with maize silage were analysed with emphasis on process acidification due to the lack of trace elements (TE), primarily cobalt and secondly sodium (Munk et al. 2010). The fermenters were operated in flow-through and had a working volume of 32 L. Two fermenters initially served as control (fermenter B2, Figure 1, and B3), whereas the others were supplied with differently concentrated TE cocktails to compensate for micronutrient lack after the first acidification. The controls had to be supplemented by TE after the second process breakdown (Figure 1). More details on the fermenters, their management and their process performance are found in Lebuhn et al. (2008) and Munk et al. (2010).

The protocol for RNA extraction was established after the experiments with the maize-fed fermenters treated with TE were finished. In order to evaluate the MQ and to determine the methanogenic activity by quantification of specific mcrA/mrtA-transcripts another experiment was started. Samples (>1 L) were taken from a 3.5 m³, mesophilic pilot-scale fermenter (SB1, Figure 2) fed with maize silage (Krapf et al. 2011). Before the experiment started, the fermenter was TE supplemented and starved for 43 days to degrade accumulated acids built up by cobalt deficiency caused by process acidification. After a single shock-load event with maize-silage at an OLR of ca. 35 kg volatile solids (VS) × m⁻³ at one day, feeding was discontinued again to recover the process.

Gas production was measured with a gas meter. The components CH₄ and CO₂ in the biogas were determined continuously by infrared gas analysis, and O₂, H₂ and H₂S by electrochemical sensors using an AWITE gas analyzer. Gas production was normalised to standard temperature pressure (STP). The ratio of total volatile acids to total inorganic or alkaline carbonate (TVA/TIC) was determined by 2-point titration basing on the work of McGhee (1968). Samples for chemical and molecular biological analyses were taken before the shock-load and 1, 2, 23, 30, 36, 50 and 58 days thereafter. Samples for transcript analysis were taken to the laboratory without temperature change within 2 hours and processed immediately. The concentration of short chain fatty acids (SCFA, C2–C7) was determined by gas chromatography, as described earlier (Munk et al. 2010).

**Nucleic acid extraction, qPCR and RT-qPCR**

Fermenter samples were homogenised (Ultra-Turrax) and washed twice in 0.85% potassium chloride (KCl). DNA
was extracted from 40 µL of washed sample using the Fast-DNA™ SPIN Kit for Soil (MP Biomedicals) and quantified by quantitative real-time PCR (qPCR). The protocols can be found in detail in Munk et al. (2010). Standard spiking with different microbes resulted in DNA recovery rates of >90% with the given isolation procedure (Garcés et al. 2005). Primers derived from an alignment containing ca. 4,000 mcrA and mrtA sequences were first published by Bauer et al. (2008).

Extraction of RNA was based on phenol/chloroform purification with lysing buffer containing GITC (total RNA extraction with Fast RNA® Pro Soil – Direct Kit, MP Biomedicals) according to the manufacturer’s protocol with the following modifications: (i) 500 µL fresh homogenised fermenter sample (FM) were washed twice with 0.85% KCl, and 40 µL washed FM were used for subsequent extraction; and (ii) an additional ethanol (70%) washing step was introduced after the isopropanol precipitation of the nucleic acids. Eluted RNA (20 or 40 µL) was immediately treated with DNase (TURBO DNA-free™ Kit, Ambion) according to the manufacturer’s protocol to remove co-extracted genomic DNA. Extracts were placed on ice for subsequent analysis or frozen (-80°C).

cDNA synthesis was carried out using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Invitrogen) in a 20 µL reaction volume containing 5 µL DNase treated RNA, reverse primer (600 nM), dNTPs (1 mM), DTT (5 mM), RNase OUT™ (40 U) and ThermoScript™ RT (15 U). Reverse transcription was performed in an Analytik Jena FlexCycler at 57.5°C for 60 min and stopped by heat inactivation at 85°C for 5 min.

1 µL of cDNA or DNA solution was quantified by qPCR in triplicates using primers for the functional gene mcrA/mrtA (MeA-i 1046f: 5′-TAYATGWSiGGH-GGIGTIGGITTYAC-3′ and MeA-i 1435r: 5′-TGRTCYTGIARRTCRWAICCRWA-GAAICC-3′) and an internal prequantified mcrA standard (Munk et al. 2010). Dilution series of extracts were analysed for PCR inhibition by checking for correct slope (2.8 to 3.8), amplification efficiency (85–125%) and y-intercept (34–38). Reactions without DNase treatment, without reverse transcription (RT) and without template served as controls for specific cDNA quantification. One Cq-value was subtracted for the calculation of the cDNA copies to compensate for the first PCR cycle in which the DNA–RNA hybrid is transformed into double-stranded DNA. The ratio of produced cDNA to measured DNA was calculated as a parameter for specific transcription activity.

In a fermenter sample spiking experiment with defined copies of an RNA virus in serial dilutions, the RNA recovery rate with the given protocol was 30% (unpublished data). This rate was used to calculate the concentration of mRNA-copies in the fermenter samples.

**Metabolic quotient**

In Munk et al. (2010), a correlation between methane productivity and the concentration of methanogens, as determined by qPCR analysis, in efficiently performing mesophilic fermenters fed with maize silage has been described. Based on a residual analysis of the model an exponential function was chosen which considers growth and a maximum concentration of the methanogenic cells in the sludge. Because of additionally analysed fermenter
samples the function \( y = 1.26 \ln(x) + 8.46; \ R^2 = 0.65; \) \( x:\) methane productivity \( [\text{L}_{\text{STP}} \ \text{CH}_4 \times \text{L}^{-1} \times \text{d}^{-1}]\), \( y:\) concentration of methanogens \( [\text{log cells} \times \text{mL}^{-1} \ \text{FM}]\) differs negligibly from the last published. The standard curve allows calculation of an expected concentration of methanogenic \textit{Archaea} at a given methane productivity assuming that the process is running efficiently. The curve shows a logarithmic dependency between methanogen concentration and methane productivity, and suggested an upper limit of approximately \(5 \times 10^9\) methanogenic \(\times\) \text{cells} \times \text{mL}^{-1} \ \text{fresh mass (FM)}\) when no substantial stress symptoms (\text{TVA/TIC} \ < 0.7; \ moderate SCFA accumulation) were obtained. The specific activity (SA), i.e. the produced methane per methanogenic cell and time, was calculated by dividing the actual methane productivity by the corresponding concentration of methanogenic \textit{Archaea}. The predicted specific activity (\(\text{SA}_{\text{pred}}\)) was determined with the data of the actual methane productivity and the corresponding predicted concentration of methanogens calculated by the standard function. The ratio of the actual measured SA (\(\text{SA}_{\text{act}}\)) to \(\text{SA}_{\text{pred}}\) (or the ratio of predicted to actual concentration of methanogens) was defined as the MQ.

**RESULTS AND DISCUSSION**

**Sample inhomogeneity and nucleic acid extraction**

The extraction of nucleic acids is crucial for reliable and reproducible results. In order to evaluate the variation introduced by sample inhomogeneity, DNA of a visually homogeneous fermenter sample was extracted in five parallels. A relative mean deviation (RMD, mean difference of data points divided by the mean) of 23% was determined. Besides, seven inhomogeneous fermenter samples were extracted twice. For these samples, a RMD of approximately 70% was obtained. In contrast, the analytical RMD, i.e. the reproducibility of different qPCR runs for methanogenic \textit{Archaea} of one extract was only ca. 10%.

Besides providing representative samples, the most critical step in the analytical procedure is hence efficient homogenisation of the samples. Large-scale or multiple-sample processing would greatly improve the reliability of the results.

**qPCR and metabolic quotient**

The concentration of methanogenic \textit{Archaea} was determined in six mesophilic maize-fed fermenters. The fermenters were differently treated with trace element solutions or cocktails (Munk et al. 2010). Different process states, from highly performing (high methane productivity up to \(2 \text{L}_{\text{STP}} \ \text{CH}_4 \times \text{L}^{-1} \times \text{d}^{-1}\) and TVA/TIC ratio \(< 0.7\)) to heavily acidified (no methane productivity with TVA/TIC ratios \(> 0.7\); Figure 1) were sampled. The concentration of methanogen DNA was lowest \((1 \times 10^5 - 5 \times 10^6 \text{cells} \times \text{mL}^{-1} \ \text{FM})\) when fermenters were acidified and/or not fed, whereas at stable high performance operation, methanogen concentrations were highest \((up \ to \ 1 \times 10^{10} \text{cells} \times \text{mL}^{-1} \ \text{FM}; \ Munk et al. 2010). The results are in the same range as, e.g. those of Yu et al. (2005). Steinberg & Regan (2009) found \(1 - 5 \times 10^6\) \text{mcrA-copies} \times \text{mL}^{-1} \ \text{in mesophilic fermenters fed with manure, bedding and milk parlor wash water, substrates that can support only poor microbial growth. Similarly low concentrations of methanogens were found at the low-performance process states of the six maize silage-fed fermenters.}

In the six fermenters, the specific methanogenic activity (SA), the activity of a single methanogenic cell, ranged between 1 and \(919 \text{pL}_{\text{STP}} \ \text{CH}_4 \times \text{methanogen}^{-1} \times \text{d}^{-1}\), depending on the fermenter state. At stable process stages, the SA range was much more narrow \((1 - 100 \text{pL}_{\text{STP}} \ \text{CH}_4 \times \text{methanogen}^{-1} \times \text{d}^{-1}\)), indicating that SA increased at stress symptoms, whereas SA decreased at acidified process states without feeding, starvation and dormancy. As an example, in fermenter B2 (Figure 1), three acidification events occurred during its operation. The development of the MQ values was compared with that of the TVA/TIC ratio (Figure 1) which is an established parameter of process stability in practice biogas plant process monitoring (Voss et al. 2009). TVA/TIC ratios below ca. 0.4 indicate that the process is stable and at about 0.7, a critical threshold is reached. Higher ratios indicate process acidification. Remarkably, the MQ peaked before TVA/TIC maxima (1st, 2nd and 3rd acidification) were obtained. High MQ values \((> 3\) were mostly obtained before the TVA/TIC exceeded 0.7 (Figure 1). This was also true for the other five fermenters (not shown). Because of biological and analytical variability, MQ values between 0.1 and 3 \(\text{(range between the dashed lines in Figure 1)}\) are considered as lying within the range of variation around the value of 1 for standard specific activity.

At the first (less severe) acidification because of cobalt deficiency (Munk et al. 2010), a small increase of the MQ to slightly above 3 was observed (Figure 1). Feeding was stopped to recover the process, and the MQ decreased to 0.03 because a relatively high number of methanogens \((1.7 \times 10^7 \text{cells} \times \text{mL}^{-1})\) was still present but inactive, as confirmed by extremely low methane productivity. When
feeding was restarted and fresh substrate was provided, the MQ increased rapidly (Figure 1) probably due to microbial growth and stress metabolism until the second (severe) acidification event, where the process collapsed completely and had to be stabilised by slaked lime. Before the acidification peak, an MQ of 23 was determined. During the second acidification, feeding was stopped again, and the MQ dropped to the extremely low level of $1 \times 10^{-6}$ (Figure 1). When the process recovered only after trace element addition (arrows in Figure 1) and inoculation with fresh fermenter material, the MQ increased to ca. 1 at stable fermenter operation (Figure 1). Before the third acidification, most likely due to lack of sodium which was not provided in the TE supplementation (Munk et al. 2010), the MQ increased again to ca. 10, before the rise of the TVA/TIC ratio indicated process disturbance. MQ analysis of the other five mesophilic maize-fed fermenters resulted in values between ca. 0.17 and 2.7 for stable process states. At acidified or inhibited process states or low activity, when the fermenters were not fed, MQ values were below this range (down to 0.0002).

If the MQ is <1, more methanogens than predicted for standard activity are present. When the microorganisms were not fed with fresh substrate, methanogenic Archaea were not provided with enough substrate (acetate, $H_2$, $CO_2$) for intense methane production. The less active the cells are, the lower the MQ becomes until cell death.

In contrast, high MQ values (up to 48.5) appeared before acidification. The MQ rose when the microbial community was growing intensively, e.g. after feeding reprise. If the MQ was >1, less methanogens than predicted were present, $SA_{act}$ was relatively high, MQ >3, and stress metabolism was prevailing.

Most importantly, the MQ increased before the TVA/TIC value, which is established as early chemical indicator of process acidification in the biogas industry. This suggests that the MQ could be even more suitable as early warning system of process acidification than the TVA/TIC ratio.

**RT-qPCR, cDNA/DNA ratio and MQ**

If microorganisms are quantified by PCR-based techniques, an unknown DNA portion from dormant or dead cells may be detected. This can particularly be true at adverse conditions (Lebuhn et al. 2005; Garcés et al. 2009) and lead to overestimation of the active population at process disturbance, especially when the ecosystem metabolic turnover rate is low. In order to detect exclusively viable organisms, the transcriptional activity of methanogens was determined. Total RNA of fermenter samples was extracted, digested with DNase and reverse transcribed with the specific $mcrA/mrtA$-primer. cDNA was quantified by qPCR using the internal $mcrA$ standard.

The mesophilic pilot-scale fermenter SB1 digesting maize silage (Krapl et al. 2011) was first starved for 43 days after acidification due to cobalt deficiency and then shock-loaded by a single feeding with an OLR of 35 kg VS $\times m^{-3} \times d^{-1}$. Methane productivity (Figure 2) increased immediately from 0.1 to $0.8 L_{STP} CH_4 \times L^{-1} \times d^{-1}$ in the first two days. Thereafter, it decreased rapidly to ca. $0.01 L_{STP} CH_4 \times L^{-1} \times d^{-1}$. The short chain fatty acids (SCFA) increased from below $0.2 g \times L^{-1}$ FM to ca. $11 g \times L^{-1}$ FM and the TVA/TIC ratio from 0.3 to 2.9. After one month of subsequent starving, the fermenter recovered from acidification and produced up to $0.5 L_{STP} CH_4 \times L^{-1} \times d^{-1}$ with SCFAs still being at a high level of $9.9 g \times L^{-1}$ FM, and another week later at $7.7 g \times L^{-1}$ FM. Propionic acid immediately accumulated to $4 g \times L^{-1}$ after the shock-load and remained at this level without major degradation throughout the experiment.

The concentration of the methanogens was at a normal level and remained almost constant during the whole shock-load experiment (between $5 \times 10^7$ and $1 \times 10^8$ methanogens $\times mL^{-1}$ FM; Figure 2(a)) indicating that no major growth of methanogens occurred. During starvation, a relatively high number of inactive cells were present, as indicated by the low MQ value of ca. 0.005 (Figure 2(b)). After the shock-load, the MQ increased to 0.9 (normal range) and decreased to a low activity level (MQ < 0.1) subsequently. The MQ did not exceed a level of 1, and there was no hint for stress metabolism of the methanogens although excess acids were produced. The concentration of methanogens appeared to be adequate for the observed methane productivity maximum of $0.8 L_{STP} CH_4 \times L^{-1} \times d^{-1}$ (Figure 2). There was no lack of trace elements (TE) during the experiment, the methanogens did not show TE deficiency stress symptoms: the MQ did not exceed the above described threshold for stress metabolism (MQ > 3), whereas it had increased up to 8 at cobalt deficiency in this fermenter in the previous experiment (not shown).

In contrast to the findings with DNA, a correlation between methane productivity and the concentration of mRNA-copies of methanogenic Archaea was evident (Figure 2(a)). Before the shock-load, only $5.5 \times 10^7$ mRNA-copies $\times mL^{-1}$ FM were detected because the fermenter was starved and accordingly, transcription activity of the methanogens was low. One day after the shock-load, a 2 log increase of $mcrA/mrtA$ mRNA-copies up to $3.5 \times 10^9$ per mL FM was determined. The feeding provided fresh...
substrate for the bacteria and for the methanogenic *Archaea* downstream in the food chain. Consequently, the activity of the methanogens, which were present at apparently sufficient numbers, increased.

The shock-load induced massive acid production, and this appeared to inhibit the methanogenic activity readily, as suggested by the sudden decline of methane productivity and the cDNA/DNA ratio (Figure 2(b)). Until the degradation of the accumulated acids, which transitionally increased the cDNA/DNA ratio and the methane productivity (Figure 2(b), days 48–78), methanogenic activity was very low without feeding.

The concentration of short-lived mcrA/mrtA-transcripts and the development of the cDNA/DNA ratio (Figure 2(b)) reflected the activity state of methanogens in the fermenter. The cDNA/DNA ratio, informing on the specific transcriptional activity of methanogens, i.e. the produced mRNA-copies per methanogenic cell, behaved very similarly to the concentration of cDNA alone because the amount of DNA was almost constant (Figure 2(a)). Information on the DNA concentration is necessary: quantification of cDNA alone would not provide enough information on the specific activity of microbes because the quantified mRNA can be produced by a low or a high amount of microbial cells in the sample.

So far, only studies on peat soil using mRNA as the target to determine the methanogenic activity were found. Freitag & Prosser (2009) investigated the transcriptional activity of methanogens in peat soil quantifying the mcrA/mrtA-gene and −mRNA. They found $8.5 \times 10^6$–$6.7 \times 10^7$ copies mRNA x g$^{-1}$ soil and reported an mcrA/mrtA transcript/gene ratio between 1 and 2. Watanabe et al. (2009) analysed mcrA/mrtA-genes and transcripts in Japanese paddy field soil under flooded and drained conditions. They found $3.7 \times 10^5$–$2.7 \times 10^6$ copies mRNA x g$^{-1}$ soil and reported an mRNA/DNA ratio of 0.8 to 1.0. In our study, cDNA/DNA ratios from $7.7 \times 10^{-5}$ to $3.5 \times 10^{-1}$ and an almost constant DNA concentration of ca. $1 \times 10^8$ methanogens x mL$^{-1}$ were determined. The higher concentration of methanogens in the fermenter compared with the paddy field soil samples is most probably due to the typically higher concentration of readily degradable substrate allowing more intense microbial growth and residually higher dormant cell densities even at famine periods, where the cDNA/DNA ratio consequently was considerably lower.

This suggests that the results for the MQ and the methanogenic activity targeting mcrA/mrtA mRNA accurately reflected the different activity states encountered in the biogas process with maize silage.

Further research will focus on the optimisation of the RNA extraction efficiency. In addition, it has to be evaluated in how far the approaches metabolic quotient and activity determination by transcriptional profiling can also be applied for other substrates and process conditions.

Our first results are promising for the development of a quick and reliable activity determination system for methanogenic *Archaea*. They suggest that the mRNA approach and the metabolic quotient can improve stress prediction in biogas process monitoring, particularly in combination with physical/chemical parameters.

**CONCLUSIONS**

Representative sampling and efficient homogenisation of samples are of major importance along with high nucleic acid extraction efficiency and PCR suitability of the extract to generate meaningful results by qPCR and RT-qPCR based approaches. Results from the activity determination of methanogenic *Archaea* in biogas fermenters show that by quantification of mcrA/mrtA-DNA without a further activity parameter, the concentration of active methanogenic *Archaea* is not always described correctly. For example, at process disturbance and when the ecosystem metabolic turnover rate is low, a large fraction of cells with low or without metabolic activity can lead to biased interpretation. Moreover, effects of induced activity and stress are not adequately reflected.

As a new parameter to determine the specific activity of the methanogens, the MQ, was developed. Assessing the MQ can serve as an early warning system for process failure as it indicated stress symptoms before the well established TVA/TIC value. The activity standard function is based on data from mesophilic maize-fed fermenters.

The cDNA/DNA ratio, which also includes an activity parameter by targeting specifically mRNA gene transcription, can also inform on the physiological state of a distinct guild. We could show this for the methanogens in a mesophilic biogas fermenter fed with maize silage.

Upcoming research will deal with a higher temporal resolution and differently operated (other temperatures, substrates) fermenters. Furthermore, it has to be proved if the MQ and the cDNA/DNA ratio can be applied for practice biogas plants, and when only a short time series or very few fermenter samples can be analysed to determine the activity state of the process. These molecular biological parameters can, particularly in combination with chemical and physical parameters, help operators of biogas plants to
better control the fermentation process and counteract imminent process failures before serious economic losses arise.

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