

Primer evaluation and adaption for cost-efficient SYBR Green-based qPCR and its applicability for specific quantification of methanogens

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Abstract In the present study nine promising primer sets, targeting Archaea and methanogenic Archaea in particular, were evaluated in silico, in vitro and in situ concerning specificity, accuracy and applicability in end-point (ep-) and especially quantitative (q-)PCR research. The main goal was to adapt and evaluate already adapted primer sets, which were partially designed in combination with TaqMan probes, in substantially cheaper SYBR Green-based qPCR applications. An initial 16S rRNA gene bank-based in silico evaluation revealed high coverage potentials for all primers within targeted groups, ranging from 71 to 90 %, except the *Methanosaeta* specific set showing a low potential of 37 %. Mentionable cross-reacting potentials could be detected for the *Methanothermobacter*, Methanomicrobiales and *Methanoculleus* sets. The in vitro evaluation with selected reference organisms revealed a specific behavior for most primer sets, while the *Methanosarcina* and *Methanothermobacter* sets showed most problematic cross-reactions in epPCR application. We were able to show that primers for detecting the total archaeal community, methanogenic orders Methanosarcinales, Methanobacteriales, Methanococcales and the genus *Methanoculleus* performed in a highly specific way and allowed an accurate quantification of targeted organisms without the use of expensive TaqMan probes. However, primer pairs designed for detecting Methanomicrobiales, *Methanothermobacter*, *Methanosarcina* and *Methanosaeta*

are not suitable for SYBR Green applications. The reliability of in situ quantifications was assessed for a typical methanogenic community, derived from a thermophilic fermenter, and confirmed via denaturing gradient gel band quantification and sequencing. Thereby, we revealed high abundances of methanogenic Archaea, mainly comprising *Methanoculleus* and Methanosarcinales, while Methanobacteriales only formed a minor fraction.

Keywords qPCR · SYBR Green · Primer · Specificity · Methanogens

Introduction

The detection and quantification via quantitative PCR (qPCR) is a promising tool to reveal conditions and alterations within microbial communities. For a huge variety of microbial groups target-oriented primers have been developed (Franke-Whittle et al. 2009; Yu et al. 2005). Nevertheless, primers can be regularly seen (re-)evaluated or proved as non-specific or not universal (Baker et al. 2003). By default, primers are designed and evaluated in silico, by aligning them to a representative quantity of sequenced target organisms in online databases (Abd-Elsalam 2003). The primer design process is a first approach but does not represent an overall reliable evaluation, since these databases are neither immune to errors nor complete and cannot simulate 100 % realistic reaction behavior of a PCR. Neither an in vitro evaluation of primers via specificity studies (pure-culture DNA from known reference organisms) nor in situ (applying complex DNA from various environments) is encompassing. However, this is the only way to gather sufficient information about whether a certain primer pair can be applied to a particular habitat.

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Otherwise, non-specific primers will lead to significant altered results, which may not represent the reality within the community.

Today's most established detection systems in qPCR applications are the amplicon quantification with the dsDNA-binding SYBR Green dyes and the more specific approach via a third detection probe (e.g., TaqMan probes) (Zhang and Fang 2006). However, these latter detection systems are substantially more expensive than SYBR Green-based systems. Furthermore, SYBR Green reaction mixes can in principle be applied to every desired primer-pair, while probes can only be used in combination with a certain primer pair.

The aim of the present study was to evaluate the specificity of a representative repertory of nine commonly used primer pairs for detecting and quantifying the most relevant members within methanogenic communities in end-point (ep)PCR and quantitative (q)PCR and their adaptability to SYBR Green-based qPCR applications. Although primers targeting functional genes are available, most of these primers aim at higher-ranking taxonomic units, like *mcrA* gene specific primers do. The *mcrA* gene encodes for a peptide within the methyl coenzyme-M reductase, the enzyme complex which catalyzes the coenzyme-bound methyl reduction and subsequent release of methane, which is thought to be common in all methanogens (Luton et al. 2002). We focused on 16S rRNA-coding genes and corresponding primer sets in order to discriminate between closely-related methanogenic taxa. All primers were checked in silico, in vitro and in situ to assess their suitability in studies dealing with complex methanogenic environments. The reference organisms for the in vitro evaluation were chosen according to results of former studies (Lins et al. 2010; Malin and Illmer 2008; Wagner et al. 2011), under the terms of a potential occurrence within diverse methanogenic habitats and with regard to cover a wide phylogenetic range. The main goal of this study is not to devalue other researchers' work. However, considering that previously published papers are supposed to present information that is usable without further evaluation and that previously published primers and adaption methods are seldom questioned, the aim of this paper—besides the strict evaluation of methanogenic primers—is to evaluate the praxis of primer adaption from TaqMan to SYBR Green qPCR assays and to question insufficiently evaluated primer publications.

Materials and methods

To differentiate between a quantitative real-time (q)PCR and a conventional PCR, and in terms of confirmability, we will refer to it as end-point (ep)PCR.

In silico evaluation of primers—phylogenetic coverage potential

The selection of the primer pairs was based on extensive preliminary paper research, choosing the most promising and up-to-date primers developed for investigating methanogenic habitats. Table 1 lists the primers to be evaluated in this study and their specifications. To reveal potential self-annealing or dimer formation, primers were at first checked with AutoDimer software (version 1.0) (Vallone and Butler 2004). Following that, primers were analyzed in silico with the TestPrime 1.0 application (Klindworth et al. 2013) using the SILVA SSU r114 RefNR database (Quast et al. 2013), available at the SILVA homepage (<http://www.arb-silva.de>). This tool makes it possible to align the sequences with verified prokaryotic and eukaryotic sequences in the SILVA comprehensive ribosomal RNA databases and evaluates their performance by simulating a virtual polymerase chain reaction (PCR) (Pruesse et al. 2007). In line with the developer's instructions, and to simulate most realistic PCR behavior, stringency parameters were set as follows: maximum number of mismatches = "1 mismatch", length of 0-mismatch zone at 3' end = "5 bases"; with exception of the Archaea- and Methanomicrobiales-specific primer sets, where stringency conditions had to be set to "0 mismatch" to receive reliable results (Table 2). We evaluated the coverage potential within most important methanogenic groups and in particular of those groups that were most important with regards to our research objects (e.g., anaerobic fermenters and soil). The database dimensions of evaluated groups were: 10581 (Arc, Archaea), 879 (MSL, Methanosarcinales), 332 (*Mesae*, *Methanosaeta*), 379 (*Methanosarcinaceae*), 188 (*Mesa*, *Methanosarcina*), 23 (*Methanomethylovorans*), 559 (MMB, Methanomicrobiales), 136 (*Mecu*, *Methanoculleus*), 73 (*Methanospirillum*), 592 (MBT, Methanobacteriales), 115 (*Methanobacterium*), 29 (*Meth*, *Methanothermobacter*) and 61 sequences in case of MCC (Methanococcales).

In vitro evaluation of primers—specificity and accuracy

The term specificity addresses the extent to which a certain primer pair in a certain assay is able to detect its targeted phylogenetic group only, while the term accuracy addresses the ability of a certain primer pair/assay to detect the exact number of individuals given.

To evaluate the in vitro specificity of chosen primers, mesophilic and thermophilic reference organisms were selected, covering the major groups of methanogenic Archaea and reflecting typical anaerobic habitats: *Methanosarcina acetivorans* (*Meac*, DSM No. 2834), *Methanosarcina barkeri* (*Meba*, DSM No. 800), *Methanosarcina thermophila* (*Mela*, DSM No. 1825), *Methanomethylovorans thermophila* (*Meme*, DSM No. 17232) and *Methanosaeta concilii* (*Meco*, DSM No.

Table 1 Characteristics of primers evaluated concerning specificity and applicability for the quantification of methanogenic archaea in SYBR Green-based qPCRs

Set	Primer	Sequence	Target	Amplicon-length (bp)	Original application	First SYBR Green adaption/evaluation	Specific & accurate SYBR Green quantification ^a
Arc	787_F	ATT AGA TAC CCS BGT AGT CC	Archaea	273	TaqMan (Yu et al. 2006)	This study (both)	Yes
	1059_R	GCC ATG CAC CWC CTC T					
MSL	812_F	GTA AAC GAT RYT CGC TAG GT	Methanosarcinales	354	TaqMan (Yu et al. 2006)	(Yun et al. 2006)/ this study	Yes
	1159_R	GGT CCC CAC AGW GTA CC					
Mesa	240_F	CCT ATC AGG TAG TAG TGG GTG TAA T	<i>Methanosarcina</i>	366	SYBR Green (Franke-Whittle et al. 2009)	(Franke-Whittle et al. 2009)/this study	No
	589_R	CCC GGA GGA CTG ACC AAA					
Mesae	MS1b_F	CCG GCC GGA TAA GTC TCT TGA	<i>Methanosaeta</i>	250	TaqMan (Shigematsu et al. 2003)	(Franke-Whittle et al. 2009)/this study	No
	SAE835_R	GAC AAC GGT CGC ACC GTG GCC					
MMB	282_F	ATC GRT ACG GGT TGT GGG	Methanomicrobiales	506	TaqMan (Yu et al. 2006)	This study (both)	No
	832_R	CAC CTA ACG CRC ATH GTT TAC					
Mecu	298_F	GGA GCA AGA GCC CGG AGT	<i>Methanoculleus</i>	308	SYBR Green (Franke-Whittle et al. 2009)	(Franke-Whittle et al. 2009)/this study	Yes
	586_R	CCA AGA GAC TTA ACA ACC CA					
MBT	857_F	CGW AGG GAA GCT GTT AAG T	Methanobacteriales	343	TaqMan (Yu et al. 2006)	(Tatara et al. 2008)/this study	Yes
	1196_R	TAC CGT CGT CCA CTC CTT					
Meth	410_F	CTC TTA ACG GGG TGG CTT TT	<i>Methanothermobacter</i>	276	SYBR Green (Franke-Whittle et al. 2009)	(Franke-Whittle et al. 2009)/this study	No
	667_R	CCC TGG GAG TAC CTC CAG C					
MCC	495_F	TAA GGG CTG GGC AAG T	Methanococcales	337	TaqMan (Yu et al. 2006)	This study (both)	Yes
	832_R	CAC CTA GTY CGC ARA GTT TA					

The last column lists the recommendation concerning the application of evaluated primer pairs in SYBR Green assays. The selected primers target 16S rRNA genes and cover a wide phylogenetic range of relevant taxa common in various methanogenic habitats, like biowaste fermenters
F forward primer, R reverse primer

^a With adaptations of the current study

2139) from the Methanosarcinales (MSL); *Methanoculleus bourgensis* (Mebo, DSM No. 3045), *Methanoculleus thermo-philus* (Meph, DSM No. 2373) and *Methanospirillum hungatei* (Mehu, DSM No. 864) from the Methanomicrobiales (MMB); *Methanobacterium thermaggregans* (Meag, DSM No. 3266), *Methanobacterium formicicum* (Mefo, DSM No. 1535), *Methanothermobacter thermautotrophicus* (Meth, DSM No. 3720) and *Methanothermobacter wolfeii* (Mewo, DSM No.

2970) from the Methanobacteriales (MBT); and *Methanococcus voltae* (Mevo, DSM No. 1537) from the Methanococcales (MCC). As out-groups anaerobic as well as aerobic Gram-positive bacteria: *Listeria monocytogenes* (Limo, DSM No. 20600) and *Coprothermobacter proteolyticus* (Copr, DSM No. 5265), Gram-negative bacteria: *Methylobacter tundripaludum* (Metu, DSM No. 17260), *Escherichia coli* (Esco, DSM No. 5347) and *Thermotoga lettingae* (Thle, DSM

No. 14385), and fungal representatives: *Geotrichum klebahnii* (*Gekl*) were chosen. All prokaryotes were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) as active growing cultures or DNA in case of no or low growth yields, while the yeast was isolated out of compost (Illmer et al. 2007).

The active growing cultures were cultivated as recommended by DSMZ and 700 μL of each culture were applied for DNA extraction with the NucleoSpin Soil DNA extraction kit (Macherey–Nagel). Quality and quantity of all DNA species were checked via agarose gel electrophoresis, spectrophotometry on a NanoDrop 2000c (Thermo Scientific) as well as with PicoGreen-based fluorometric detection (Quant-iT) on a Zenyth 3100 multimode fluorometer (Anthos) to obtain highly precise DNA quantities. Afterwards the DNA extracts were diluted in DNase/RNase-free distilled water from gibco life technologies (A. d., aqua destillata) to final concentrations of 1.0 and 0.2 $\text{ng } \mu\text{L}^{-1}$ respectively before being applied in ep- and qPCR applications for evaluating specificity and sensitivity. To gain comparable information in the assays, we added equivalent amounts of genomic DNA per PCR approach. Preliminary tests showed that 0.2 $\text{ng } \mu\text{L}^{-1}$ DNA concentrations were sufficient for qPCR-based evaluation approaches, while, due to the lower sensitivity regarding epPCR/GE-based approaches, DNA concentrations had to be increased to 1.0 $\text{ng } \mu\text{L}^{-1}$ in this case.

Original and modified PCR conditions are stated in Table 3. The melting temperatures were recalculated with OligoAnalyzer software (version 3.1; Integrated DNA Technologies [<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx>]) and corresponded well to the original specifications given for qPCR applications, but not concerning epPCRs. In the case of epPCR applications, dNTPs were set to 0.2 mM, while primer concentrations were set to 0.2 (Arc, Mesa, Mesae, Meth and Mecu) or 0.4 (MSL, MCC, MBT and MMB) μM . 0.625 units of DNA polymerase (BioTherm) were added per epPCR approach. Concerning qPCR, we increased the MgCl_2 concentration to 0.5 mM, while primer concentrations were in most cases decreased to 0.15 (Mesae), 0.2 (Mesa and Meth), 0.25 (Arc, MCC and MMB) or 0.3 (MSL, MBT and Mecu) μM .

Quantitative PCRs were performed with the SensiMix SYBR No-ROX kit (Bioline) on a Corbett Life Science (Qiagen) Rotor-Gene 6000 system. All PCR reactions and approaches were performed at least in duplicates, with no template controls (NTCs), positive and negative controls. One μL of template-DNA was added per epPCR, while two μL were added per qPCR approach. The final volumes of ep- and qPCR approaches were 25 and 20 μL respectively. For quantification and efficiency calculation we used diluted standards and plotted the C_T (cycle threshold) values against the log of given templates to gain standard graphs (Bustin

et al. 2009). As standards we used purified PCR products of known concentrations. Therefore we applied pure DNA from a corresponding reference organism for each primer pair (*Meac* for Arc primer, *Meac* for MSL primer, *Meba* for Mesa primer, *Meco* for Mesae primer, *Mehu* for MMB primer, *Mebo* for Mecu Primer, *Meth* for Meth primer, *Mewo* for MBT primer and *Mevo* for MCC primer) in conventional epPCRs, purified and quantified the amplicons as described above. Subsequently, the copy numbers were calculated, based on the measured DNA amount (ng) and on the verified amplicon length (bp) as described in Yun et al. (2006). Stock solutions with $1\text{E}+09$ copies μL^{-1} were prepared, aliquoted and stored frozen. Fresh batches were prepared shortly before use. For calibration curves, dilutions from $1\text{E}+08$ to $1\text{E}+02$ were applied in duplicates (dynamic range). The efficiency calculation itself was performed by the Rotor-Gene software. From all epPCR approaches and representative spot tests of qPCR, runs were checked via gel electrophoresis. All qPCR runs were checked with melt curve analysis, with temperature intervals of 0.2 $^\circ\text{C}$, subsequent to amplification (provided as supplement 02, “In vitro qPCR”).

To draw conclusions regarding specificity, sensitivity and accuracy of the adapted qPCR applications with the selected primer sets, copy numbers of 16S rRNA genes were calculated based on added reference DNA amounts (Einen et al. 2008) and compared with measured values (Figs. 1, 2). Copy numbers per genome, of each reference organism, were obtained from the rrndb, the Ribosomal RNA Operon Copy Number Database (<http://rrndb.mmg.msu.edu>), while for non-listed species the averaged copy number of the corresponding genus was used. Organismic genome sizes could be determined for Meac (Galagan et al. 2002), Meba (Zhu et al. 2012), Mela (Diaz-Perez et al. 1997), Meco (Zhu et al. 2012), Mebo (Maus et al. 2012), Mehu (Anderson et al. 2009), Meth (Fogel et al. 1999), Mewo (Fogel et al. 1999), Mevo (Fogel et al. 1999), Limo (Fogel et al. 1999), Copr (Koonin and Wolf 2008), Metu (Dam et al. 2012), Esco (Fogel et al. 1999), Thle (Blumer-Schuette et al. 2008) and Gekl (Gregory et al. 2006), while for Meme, Meph, Meag and Mefo averaged genome numbers, characteristic for their taxon, were chosen. For more species specific information see the additional table entitled “Reference Organisms” (supplement 01).

In situ measurements and confirmation of specificity

To evaluate the performance of most specific primer sets in best adapted ep- as well as qPCR applications, an in situ evaluation was performed with samples of a typical methanogenic habitat, a thermophilic anaerobic digester. The investigated samples were obtained from the control approach of the fermenter sludge experiment described in Lins et al. (2012), where diluted fermenter sludge was

incubated in a modified DSMZ 119 medium with 150 mM acetate for 77 days in triplicates. During the experiment samples were collected at days 0, 10, 21, 31, 42, 56 and 77. DNA extraction and checks of fermenter sludge (FS) samples as well as qPCRs were carried out as described above and listed in Table 3. Temperature intervals of the melt curve analyses were set to 0.5 °C. Standard graphs and melt curves are provided as supplementary material (supplement 03, “In situ qPCR”). Following that, the overall Archaeal community was investigated using denaturing gradient gel analysis (DGGE), and a representative collection of DNA bands was excised after separation. For the DGGE analyses a GC clamp (Muyzer et al. 1993) was attached to the 5'-end of the Arc forward primer and an epPCR was conducted with the conditions described above and listed in Table 3. The DGGE protocol was altered based on the work of Sekiguchi et al. (2001). For the separation an INGENYphorU electrophoresis system was used (60 °C, 100 V for 16 h). The acrylamide concentration in the gel was between 7 and 8 %, while urea and formamide concentrations were between 35 and 70 %. After separation, DNA bands were stained with silver nitrate. Distinct DNA bands were quantified relatively. For this, densitometric curves of each DGGE lane were readout with ImageJ software (available at: <http://rsb.info.nih.gov/ij/>), after background subtraction. Afterwards, an averaged threshold was determined, and peak areas were integrated and set into relation to the sum of peak areas per lane. To obtain qualitative data, products were separated in a new DGGE and stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen). Equivalent, previously quantified bands were excised under UV light, suspended in A. d. and used as template in a new PCR with the Arc primer pair, but without GC clamp to obtain better sequencing results. Positive PCR products were purified with NucleoSpin Extract II (Macherey–Nagel) and sequenced by Eurofins MWG Operon (Ebersberg, Germany). Passed sequences were processed and checked concerning quality with CLC DNA Workbench 5.6.1 (CLC bio) and aligned via NCBI Blast tool (<http://blast.ncbi.nlm.nih.gov/>).

Finally, the quantitative data gained via qPCR were compared to the quantitative–qualitative DGGE and sequencing results to reason the reliability of SYBR Green-based qPCR adapted in this study.

Results

All primer pairs that were evaluated in the study regarding specificity, accuracy and applicability in end-point and, most importantly, in SYBR Green-based quantitative PCR applications are shown in Table 1. This includes the summary of the evaluation, giving a recommendation whether a SYBR Green-based quantification is advisable or not.

In silico evaluation of primers—phylogenetic coverage potential

Table 2 summarizes the coverage potential of chosen primers within selected methanogenic groups. While the total coverage of the Arc primers is quite low (70.5 %), the coverage within methanogenic Euryarchaeota is substantially higher, ranging from about 80 to 100 %. Furthermore, no potential cross-reactivities regarding bacteria or Eukaryota could be determined. The MSL set had a potential of 76 % to detect members within Methanosarcinales, while members of the genus *Methanosaeta* are only covered to 68 % compared to 89 % concerning *Methanosarcina*. A slight cross-reacting potential of 0.5 % is given for *Methanolinea* (MMB). The Mesa set showed a high coverage of 82.9 % within the target group and only negligible cross-reactions of 0.2 % in the C19A group (MMB) and concerning ANME-3 and *Methanolobus* (*Methanosarcinaceae*, MSL). The lowest affinity to its target group could be confirmed for the Mesae primers, detecting only 37 % of available sequences, with a negligible cross-reaction potential of 0.3 % within uncultured *Methanosarcinaceae* (MSL). A potential of 75 % for specific coverage is given with the MCC set, showing only slight false-positive detection potential for pCIRA-13 (Euryarchaeota). The MBT primer set performed in a highly specific way, covering 90 % of all Methanobacteriales, 88 % of *Methanobacterium* and 100 % of *Methanothermobacter*. However, a negligible cross-reaction potential concerning uncultured Halobacteria with 0.1 % and Thermoplasmata with 0.2 % (both Euryarchaeota) was revealed. Even though the Meth set showed highly specific coverage (83 %) within *Methanothermobacter*, a proportionally high percentage of 8 % within the closely related *Methanobacterium* species was also covered. Basically, the MMB set showed a moderate coverage of 73 % within Methanomicrobiales, 88 % within *Methanoculleus* and 79 % within *Methanospirillum*, although detecting members within *Methanosarcina* (1.4 %), *Methanosaeta* (2.3 %) and *Methanocalculus*, all belonging to the MSL order. The Mecu set highlighted a high coverage of 89 % within the targeted group. Slight cross-reactions could be detected in closely related groups of *Methanospirillum* (1.5 %), *Methanolinea*, *Methanosphaerula* and *Methanofolis* (all MMB) and members within the MSL cluster (0.4 %).

In vitro evaluation of primers—specificity and accuracy

As summarized in Table 3, additions and massive alterations compared to the original specifications were necessary to obtain reliable and specific measurements. Although we observed high efficiencies with initially

Table 2 16S rRNA gene bank based in silico evaluation of selected primers

Primer set	Phylogenetic group/coverage (%)												
	Arc [10581]	MSL [879]	Mesae [332]	Methanosarcinaceae [379]	Mesa [188]	Methanomethylivorans [23]	MMB [559]	Mecu [136]	Methanospirillum [73]	MBT [592]	Methanobacterium [115]	Meth [29]	MCC [61]
Arc	70.5	80.3	83.0	83.8	88.6	100	90.1	89.0	95.8	89.3	79.6	87.0	90.6
MSL	6.9	75.8	67.8	63.0	89.1	84.6	0.5	0.0	0.0	0.0	0.0	0.0	0.0
Mesa	1.4	16.9	0.0	41.0	82.9	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Mesae	1.1	14.0	36.9	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MCC	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	75.4
MBT	6.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	89.9	88.2	100	0.0
Meth	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.8	8.0	82.8	0.0
MMB	0.8	1.1	2.3	0.6	1.4	0.0	72.9	88.3	78.8	0.0	0.0	0.0	0.0
Mecu	1.3	0.4	0.0	0.9	2.0	0.0	27.4	89.2	1.5	0.0	0.0	0.0	0.0

The coverage potentials of evaluated primers within most relevant phylogenetic archaeal or methanogenic taxa are stated in percent. The SILVA database dimensions of the corresponding phylogenetic groups are stated in square brackets. For primer set and phylogenetic group abbreviations see Table 1. Bold underlined values emphasize the coverage potential within the targeted group(s) of the corresponding primer. The coverage potential within targeted sub-groups of a certain primer set is also stated (*bold*). Values in italic highlight in silico cross-reactions, meaning false-positives

Arc Archaea, MSL Methanosarcinales, Mesae Methanosarcinae, Mesa Methanosarcina, MMB Methanomicrobiales, Mecu Methanococcales, MBT Methanobacteriales, Meth Methanobacter, MCC Methanococcales

applying 2-step qPCR variants, most NTCs were false-positive and primer dimers were detectable. Therefore, we decided to discard 2-step assays in general and changed to 3-step variants for all primer pairs. To get rid of non-specific products and primer dimers in ep- as well as qPCRs as far as possible, drastically lower primer concentrations, higher annealing temperatures and shorter elongation times had to be applied. Although amplification efficiencies could not be increased or kept constant in all cases, clearly noticeable for Mesae and MCC primers (Table 3), corresponding qPCR conditions were chosen as the best compromise between specificity and efficiency.

All primer pairs could detect their corresponding reference organisms. Primer pairs Arc, MSL, Mesae, MBT and Mecu showed highly specific results and are therefore suitable for further epPCR-based qualitative research on methanogenic communities (e.g., PCR-DGGE-based investigations) for assessing microbial diversity and for sequencing purposes. Primer pairs Mesa, MCC, Meth and MMB revealed mentionable non-specificities, with most problematic cross reactions of the Mesa set detecting the closely related *Methanomethylivorans thermophila* (MSL) and with the Meth set detecting the closely related *Methanobacterium thermaggregans* (MBT).

Compared to the results of end-point amplifications, quantitative detection and analysis via SYBR Green-based real-time PCR revealed considerably higher non-specificities within certain primer sets, although highlighting primer sets Arc, MSL, MBT and MCC as well as Mecu to be valuable tools for specific and accurate quantification of targeted methanogens. To gain information about the sensitivity and accuracy of the primer sets used in the adapted qPCR applications, the added 16S rRNA gene copy numbers, calculated according to actually applied DNA amounts, were compared with the measured gene copy numbers in vitro. As can be seen in Figs. 1 and 2, the actually measured copy numbers of Archaea, Methanosarcinales, Methanobacteriales and Methanococcales as well as *Methanoculleus* cluster are close to the calculated, added number of copies, while non-target organisms are either not amplified (MSL) or in case of false-positives the quantification results are about 1–3 (MBT), 2.5–4.5 (Mecu) and 3.5–4.5 (MCC) orders of magnitude lower and alter the quantification result to a negligible or slight extent. In case of Archaea we observed higher non-specific reactions concerning bacterial and fungal representatives, although the averaged distance between targeted Archaea and false-positives was still 2 orders of magnitude, altering the Archaeal quantification. Although, due to the limited quantitative impact on the quantification results their appliance is acceptable, if a careful pre- and post-analytical evaluation of the results is carried out. Further information is provided in supplement 04 (“In vitro qPCR—selected primers”).

Table 3 Original and modified ep- and qPCR conditions applied for the evaluation of chosen primer sets

Primer-set	Conditions	End-point PCR			Quantitative PCR			Efficiency %
		Denaturation	Annealing	Elongation	Denaturation	Annealing	Elongation	
Arc	Original	n.a.	n.a.	n.a.	10 s, 94 °C	30 s, 60 °C		84
	Modified	45 s, 95 °C	45 s, 57 °C	45 s, 72 °C	20 s, 95 °C	20 s, 61 °C	20 s, 72 °C	81
MSL	Original	Yu et al. (2005)			10 s, 94 °C	30 s, 60 °C		89
	Modified	n.m.	n.m.	n.m.	20 s, 95 °C	20 s, 63 °C	15 s, 72 °C	84
Mesa	Original	Franke-Whittle et al. (2009)			20 s, 95 °C	20 s, 64 °C	20 s, 72 °C	71
	Modified	n.m.	n.m.	n.m.	20 s, 95 °C	20 s, 65 °C	15 s, 72 °C	71
Mesae	Original	n.a.	n.a.	n.a.	15 s, 95 °C	60 s, 60 °C		101
	Modified	45 s, 95 °C	60 s, 55 °C	45 s, 72 °C	20 s, 95 °C	20 s, 68 °C	15 s, 72 °C	78
MMB	Original	n.a.	n.a.	n.a.	10 s, 94 °C	30 s, 63 °C		82
	Modified	45 s, 95 °C	60 s, 57 °C	60 s, 72 °C	20 s, 95 °C	20 s, 60 °C	20 s, 72 °C	78
Mecu	Original	Franke-Whittle et al. (2009)			20 s, 95 °C	20 s, 65 °C	20 s, 72 °C	76
	Modified	n.m.	n.m.	n.m.	20 s, 95 °C	20 s, 65 °C	15 s, 72 °C	86
MBT	Original	n.a.	n.a.	n.a.	10 s, 95 °C	10 s, 65 °C	20 s, 72 °C	64
	Modified	45 s, 95 °C	60 s, 57 °C	60 s, 72 °C	20 s, 95 °C	20 s, 61 °C	20 s, 72 °C	81
Meth	Original	Franke-Whittle et al. (2009)			20 s, 95 °C	20 s, 61 °C	20 s, 72 °C	100
	Modified	n.m.	n.m.	n.m.	20 s, 95 °C	20 s, 65 °C	15 s, 72 °C	92
MCC	Original	n.a.	n.a.	n.a.	10 s, 94 °C	30 s, 60 °C		82
	Modified	45 s, 95 °C	60 s, 55 °C	60 s, 72 °C	20 s, 95 °C	20 s, 61 °C	15 s, 72 °C	69

Originally some qPCR programs were designed as 2-step variants, so annealing and elongation steps were combined. Cycle repetitions were between 30 and 45. In all epPCR applications an initial denaturation step of 5 min at 95 °C was applied, while the final elongation was 7 min (Arc, MSL, MCC, MBT and MMB) or 10 min (Mesa, Mesae, Meth and Mecu) at 72 °C. In all qPCR runs an initial denaturing step of 10 min at 95 °C was applied. For primer set abbreviations see Table 1

n.a. not available, *n.m.* not modified

Even though the MMB set showed a highly accurate quantification of target organisms, we were able to observe extensive cross-reactions, altering quantitative results more considerably (Fig. 1). The quantification values of primer pair Mesa showed an extremely broad distribution pattern, detecting target organisms added in concentrations of about $1\text{E}+05$ copies per reaction, in quantities of $1\text{E}+03$ to $1\text{E}+08$, therefore making their application in a SYBR Green-based qPCR not reliable (Fig. 2). Furthermore, the high quantification values of many non-target reference organisms excludes the primer pair from SYBR Green quantification. In case of the Mesae set, the low affinity to targeted *Methanosaeta* could be proved and moreover extensive cross-reactivities (Fig. 2), also excluding this primer pair. A different reaction behavior was detectable for the Meth set. Even though reference *M. wolfeii* was detected to almost 100 %, reference *M. thermautotrophicus* was detected in 2 orders of magnitude lower quantities (Fig. 2). In connection with the constant detection of all applied references in mentionable quantities, most likely due to a universal amplification behavior of the primers, a reliable quantification with the Meth set could not be proved.

In situ measurements and confirmation of specificity

Based on the results from the in silico and in vitro evaluation of all primer sets, the most specific primer pairs and the most reliable SYBR Green-based qPCR adaptations were applied for investigating an anaerobic digester community.

Highest abundances in qPCR were obtained with Arc specific primers, revealing values between $6\text{E}+07$ and $5\text{E}+09$ Archaea mL^{-1} during the fermenter sludge (FS) experiment, while the Mecu set revealed *Methanoculleus*, with $2\text{E}+07$ to $2\text{E}+09$ organisms mL^{-1} FS, to be the most abundant methanogenic genus (Fig. 3). As second-most abundant methanogens the Methanosarcinales could be addressed, with the MSL set detecting $8\text{E}+06$ to $1\text{E}+08$ organisms mL^{-1} FS, becoming the most abundant methanogens in the end of the cultivation (Fig. 3). The MBT primer pair illustrated significantly ($p < 0.001$) lower abundances of Methanobacteriales compared to Arc, Mecu and MSL after 10 days of incubation, which were between $6\text{E}+05$ and $8\text{E}+06$ mL^{-1} FS (Fig. 3). No members of the Methanococcales were detected.

Based on the semi-quantitative DGGE band determination and combined sequencing results, we were able to

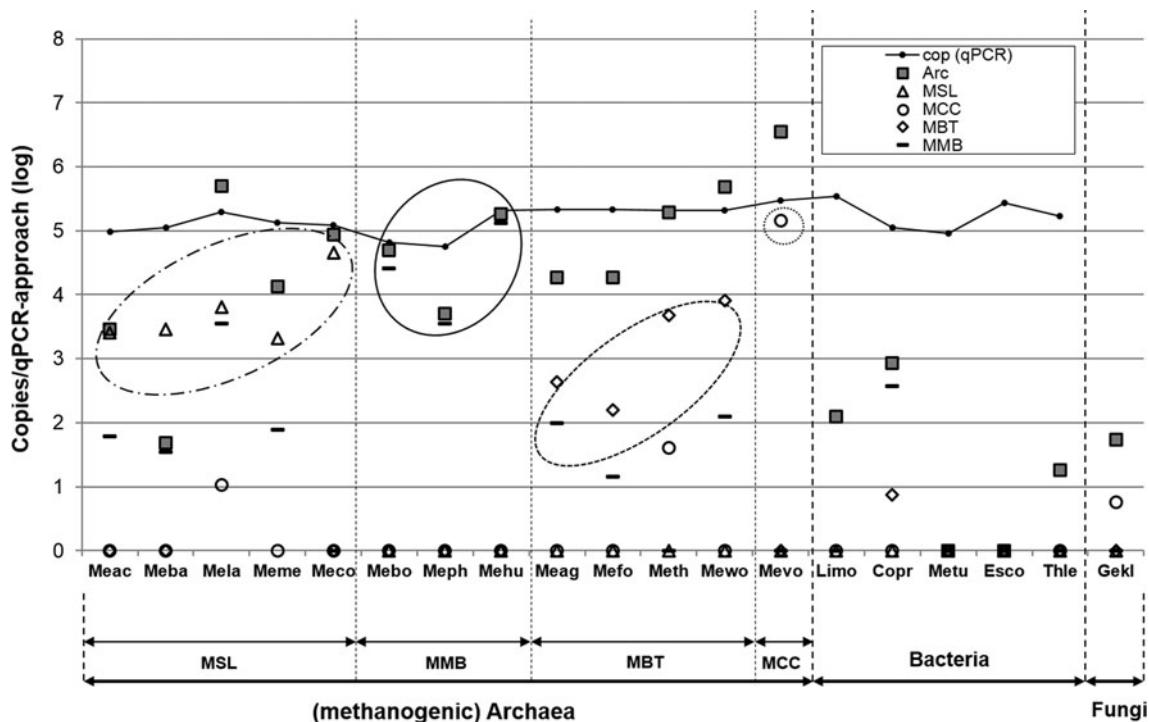


Fig. 1 Specificity and accuracy of Archaea (Arc) and methanogenic order specific primers (MSL, MCC, MBT and MMB) were checked in vitro in adapted SYBR Green-based qPCR applications. Normalized amounts of DNA from reference organisms were added per qPCR approach and corresponding 16S rRNA gene copy numbers were calculated [cop (qPCR)]. These calculations are compared with

the copy numbers actually measured, obtained from the calibrated qPCR runs with the corresponding primer sets. Encircled areas visualize the quantification of order specific primers within targeted groups. It is clear that primer pairs Arc, MSL, MCC and MBT show no or slight unspecific amplification behavior, by detecting false-positives in several orders of magnitude less than targeted groups

show that all bands amplified with the Arc primer set were of Archaeal and methanogenic origin, emphasizing the appropriateness of the Arc set for quantifying methanogens in fermenter sludge. Furthermore, we could show that members of *Methanoculleus* (MMB order) form the most abundant genus. Related bands contributed to approximately 100 % of total bands at the beginning of the incubation and decreased to about 50 % at the end (Fig. 4). Bands affiliated to members of the Methanosarcinales (basically *Methanosarcina*, with negligible *Methanome-thylovorans* contribution) raised from about 0 to 50 % during the in situ experiment (Fig. 4). No members of Methanobacteriales or Methanococcales were found, due respectively to their low abundance and absence of corresponding representatives. The relative qPCR values of MSL and Mecu, in percentage of the total Archaea, affirm the DGGE/sequencing results. The high congruence is most evident in case of the MSL increase during the incubation experiment. The trend of *Methanoculleus* is not as clear, although highlighting that Mecu form the dominant methanogenic group in the fermenter sludge experiment. While members of MBT were detected during qPCR, together with unidentified organisms contributing to the total archaeal community and the difference that is not

represented in DGGE/sequencing data. However, the main message that all detected Archaea are methanogens and the two dominating groups are *Methanoculleus* and Methanosarcinales could be shown by this unconventional approach.

Discussion

This study does not claim to present exhaustive evaluated primers, because this is not entirely feasible considering the huge variety of parameters and their combination possibilities. Primers are created on basis of described and sequenced organisms. This may lead to an underestimation of the actual abundance of a certain group due to a low or no coverage within unknown and genomic differing members within a phylogenetic group (Murrell and Jetten 2009). Complete coverage of related microorganisms with this technique is therefore unlikely. Nevertheless, the assessment and the quantification of microorganisms based on 16S rRNA genes is still a valuable and common tool (Franke-Whittle et al. 2009; Skillman et al. 2004; Tataru et al. 2008; Yu et al. 2006). Our goal was to transfer a representative set of primers for detecting methanogens

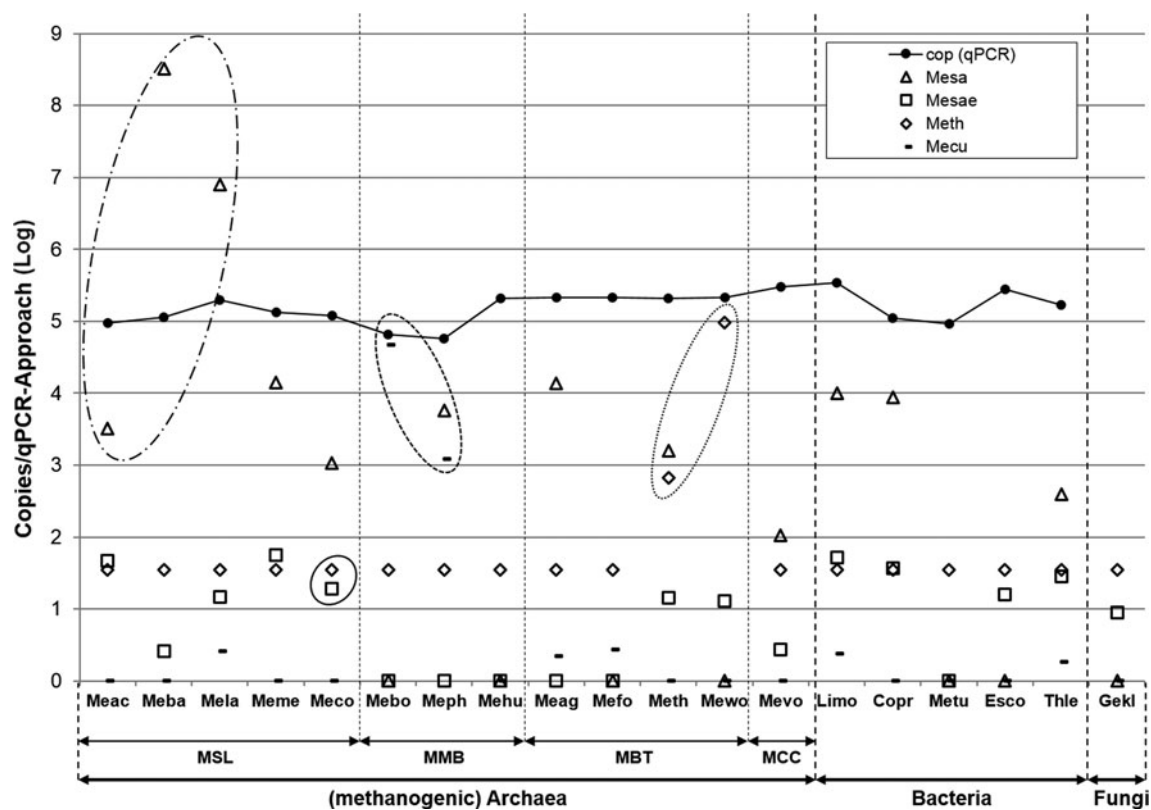


Fig. 2 Specificity and accuracy of methanogenic genus specific primers (Mesa, Mesae, Meth and Mecu) were checked in vitro in adapted SYBR Green-based qPCR applications. Normalized amounts of DNA from reference organisms were added per qPCR approach and corresponding 16S rRNA gene copy numbers were calculated [cop (qPCR)]. These calculations are compared with the copy

numbers actually measured, obtained from the calibrated qPCR runs with the corresponding primer sets. *Encircled areas* visualize the quantification of genus specific primers within targeted groups. It is clear that only primer pair Mecu showed a slight unspecific amplification behavior, by detecting false-positives in several orders of magnitude less than targeted groups

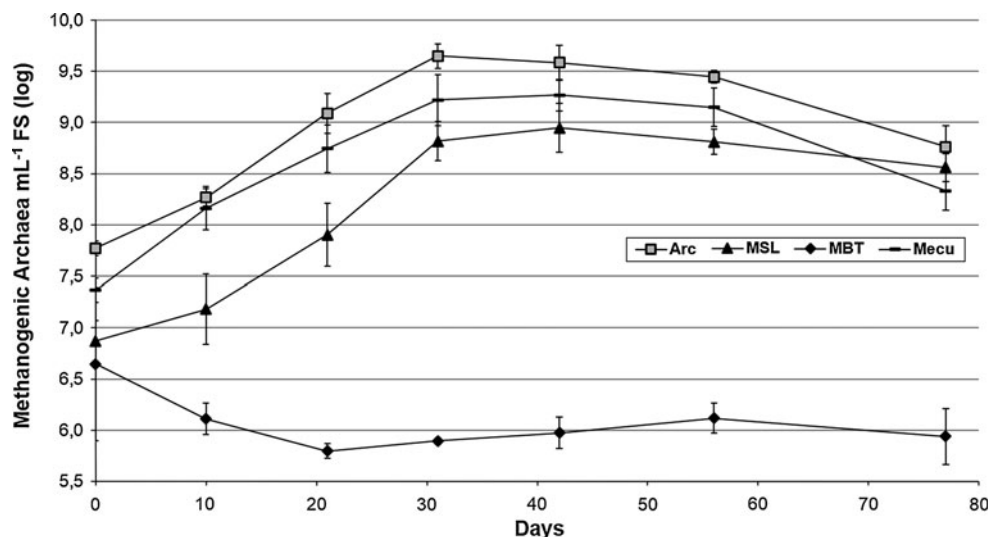
from the expensive TaqMan system, where the costs for the additional probe is about ten times higher than for a standard primer, to the more economic SYBR Green system.

After the in silico evaluation, primer pairs Mesae (due to the considerable low target-specific coverage), Meth (due its significant high coverage within a closely related group), MMB and Mecu (due to their slight, but broad physiological coverage within other methanogenic groups) were especially checked before application. The in vitro evaluation of all primers with reference organisms revealed a more distinct non-specific behavior regarding epPCR applications than expected according to the in silico evaluation. Primer sets Mesa and Meth detected closely related organisms, which was a significant drawback as cross-reacting organisms were thermophilic and likely to inhabit similar habitats as members of the desired methanogenic genera do. Although the copy numbers added and calculated and the actually measured numbers were not congruent in our in vitro qPCR evaluation, the orders of magnitude reached proved that affiliated reference organisms were detected in much higher quantities than false-positive responding organisms. These results can be

regarded as a quality criterion for accurate working primer sets in adapted qPCR applications. Based on the results we obtained by a comprehensive research approach, combining in silico, in vitro and in situ tests, we recommend the use of primer pairs Arc, MSL, Mecu, MBT and MCC in SYBR Green-based approaches for specific and accurate quantification of archaeal and methanogenic members of corresponding physiological groups, while the application of Mesa, Mesae, MMB and Meth primer sets is not suitable in SYBR Green assays.

Due to the highly specific and reliable performance of five out of nine primer sets, we applied them for in situ measurements in a typical methanogenic habitat—thermophilic fermenter sludge. Even though members of the Methanococcales have so far only been found in marine sediments (Liu and Whitman 2008), corresponding primers were evaluated in this study for potential application, although in an unlikely habitat, serving as out-group. The high congruence of qPCR and DGGE/sequencing results by investigating fermenter sludge samples convinced us of the specificity and accuracy of these sets in combination with our adaptations of the SYBR Green-based detection

Fig. 3 The quantitative development of the total archaeal community (Arc)—representative for the total methanogenic community in the anaerobic batch experiment with fermenter sludge—and of the orders Methanosarcinales (MSL) and Methanobacteriales (MBT), as well as the genus *Methanoculleus* (Mecu) is displayed in course of 77 days of incubation in an acetate containing medium



system. The occurrence of Methanomicrobiales (*Methanoculleus* sp.) and Methanosarcinales (*Methanosarcina* sp.) in significant abundances in the investigated habitat is additionally confirmed by previous studies (Lins et al. 2010; Malin and Illmer 2008; Wagner et al. 2011), supporting the qPCR-based quantification results. Even though we could not prove members of the Methanobacteriales at species level by DGGE/sequencing in the in situ measurements, while detecting them with qPCR, previous studies dealing with the same sludge were able to do so, detecting *Methanothermobacter* species in distinct abundances (Malin and Illmer 2008; Wagner et al. 2011), and supporting current MBT detection.

In this investigation we did not consider the fifth known class of methanogenic Archaea, the Methanopyrales, because their only approved habitat are marine geothermal sediments and growth temperatures of about 98 °C (Liu and Whitman 2008).

Due to the fact that all large investigated methanogenic groups (the Methanosarcinales, Methanomicrobiales and Methanobacteriales) include members that are well-known for (co-)inhabiting anaerobic digesters, cross-reactivity seems to be a central problem that might lead to significantly altered qualitative and quantitative results. Further information about environmental parameters, such as temperature, nutrient availability and phylogenetic

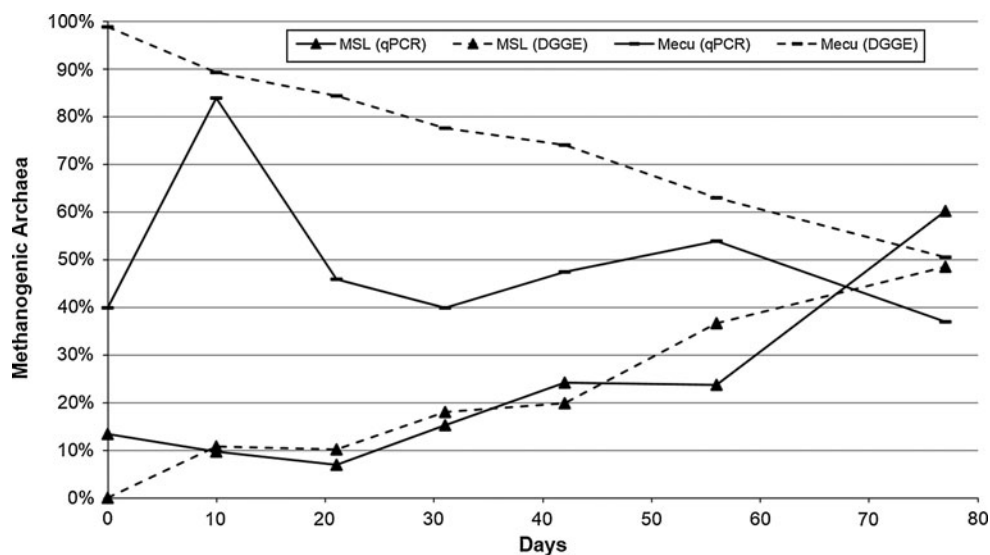


Fig. 4 The absolute percentage of the dominant methanogens, the genus *Methanoculleus* (Mecu) and the order Methanosarcinales (MSL), within the total archaeal community during the anaerobic fermenter sludge experiment, was measured via qPCR and compared

to the semi-quantitative values obtained via DGGE peak area quantification and band affiliation via sequencing, in order to emphasize the reliability of the selected primers for in situ measurements on fermenter sludge communities

affiliation, is a necessary prerequisite in order to decide whether the application of these primer pairs is appropriate.

Due to multiple operon copies in many organisms (Klappenbach et al. 2001) and limited information about methanogenic genomes (Yu et al. 2005), the determination of exact organismic numbers is also a challenge. Furthermore, operons can exist as identical copies or in different forms, sometimes with big sequence differences, which makes the interpreting the results even more difficult (Acinas et al. 2004). In some cases the change to functional gene quantification may be reasonable. However, such functional genes that are characteristic for a certain phylogenetic group are not always available or allow only a rough differentiation of a certain community. Even though in the case of methanogenic Archaea such genes are available (*mcrA* quantification) (Nunoura et al. 2008), we wanted a more detailed differentiation of this diverse community.

In conclusion, the practice of primer adaption from probe- to SYBR Green-based assays is valuable regarding cost reduction. However, a critical multifactorial evaluation of such adapted assays in silico, in vitro and in situ is essential. Furthermore, a more critical review and control process of commonly used 16S rRNA gene targeting primers regarding specificity and accuracy is needed. Primers are designed on basis of up to a current date status of online databases and may therefore become obsolete in future. However, we recommend to apply the in silico designed primers not only in combination with target organisms in vitro, but also with non-target organisms and to evaluate the appropriateness of a certain primer pair for every complex habitat they should be applied at. This is of course a challenging topic and we also came to limits we could so far not evaluate. Although, for current and future research on fermenter sludge, in combination with the designed post-analytical methodology, it is possible to draw conclusions about methanogenic developments in this certain habitat.

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