Development and application of rapid methods for quantification and cultivation of methanogens in bio-methane producing fermentors

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Abstract

This thesis describes the development of simple methods for isolation of methanogens as well as a method for microscopic quantification. Using an extensive literature review, a detailed account of the method development including modification, improvement, and validation of the technique was described. Additionally, a new indicator was consequently developed using the microscopic technique as a rapid tool for assessment of anaerobic digesters. This indicator was empirically established, based on complex interaction between methanogenic cell counts, chemical parameters and biodiversity. The indicator, so-called quantitative microscopic fingerprinting index (QMFI), was the first approach to combine microscopic cell counts with environmental factors including volatile fatty acids and biodiversity to find and objectify methanogenic activity in anaerobic digesters. Using the microscopic technique, it was found that morphological changes of methanogens could be an early and new indicator regarding temperature and ammonia perturbation as a new parameter for the monitoring of anaerobic digesters. Additionally, a simple cultivation and isolation method for strict anaerobic methanogens without a cost-intensive anaerobic chamber was developed. An anaerobic petri-dish box was developed to enable simple selection of methanogenic colonies on a single petri-dish under pressurised conditions with H_2-CO_2 as sole energy and carbon source. In this study, several methanogens were isolated from anaerobic digesters by the developed method. The development of both microscopic quantification and isolation techniques could contribute to the analysis of methanogens in anaerobic digesters as a new simple, rapid, and reliable tool to increase the process efficiency of digesters.

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Motive of the research subject

Additionally I would like to mention the motive of my research. I would just know the life secret of methanogens how they use the smallest gas 'hydrogen' and currently the most problematic gas 'carbon dioxide' in terms of global warming, as single energy and carbon source. How they are feeling well in strict anoxic condition without any light. How they restore the stinky hydrogen sulphide and ammonia gas for valuable sulphur and nitrogen source. How beautiful they shine uniquely in the darkness based on the unique coenzyme F_{420}. How they produce valuable energy carrier methane gas from useless manure and biowaste. Why they just use little amount of carbon for themselves growing and most amount for production of methane. The secret of methanogens has brought me into the research world every day. That was an amazing moment to see the methanogens under darkness in my laboratory. All such impressions on methanogens were initiated by my supervisor Prof. Paul Scherer having explained methanogens by using his specific speech style of anthropomorphism or personification of methanogens during his lecture and conversation from time to time.

Declaration Statement

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was done under the guidance of Professor [Paul Scherer[1], Steven Kelly[2]], at the Hamburg University of Applied Sciences[1] and University of the West Scotland[2].

[Yong Sung Kim]

In my capacity as supervisor of the candidate's thesis, I certify that the above statements are true to the best of my knowledge.

[Paul Scherer, Steven Kelly]

Date:

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List of Abbreviations

A	Average
AO	Acridine orange
C	Carbon
CFU	Colony forming units
DAPCO	1,4-Diaza-bicyclo (2,2,2) octane
DAPI	4,6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DTAF	5-4,6,-dichlorotriazin-2-aminofluorescein
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EEG	Renewable energy law (Erneuerbare Energie Gesetz)
fs	Stabilising factor
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
Fluo	Fluorescence
HAc	Acetic acid
HAW	Hochschule für Angewandte Wissenschaften Hamburg
HRT	Hydraulic retention time
LED	Light emitting diode
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POM	Polyoxymethylene
PMMA	Polymethyl methacrylate
PTFE	Polytetrafluoroethylene
QMF	Quantitative microscopic fingerprinting
QMFI	Quantitative microscopic fingerprinting Index
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Sulphur
SD	Standard deviation
TMCP	Total methanogenic cell power
UV	Ultraviolet
VFA	Volatile fatty acids
VS	Volatile solid

Chapter 1: Introduction

Truth is ever to be found in simplicity, and not in the multiplicity and confusion of things

Isaac Newton

Chapter 1: Introduction

1. Background

Growing concerns about energy security and the need to mitigate greenhouse gas emissions have accelerated the deployment of renewable and sustainable fuels such as bio-methane (McKendry 2002; Weiland 2006; Deublein and Steinhauser 2010). Along this trend, economic interest in bio-methane production also arose; consequently, the number of bio-methane plants increased drastically with the support of the government in the last decades in Germany. This led to concomitant, extensive studies to develop appropriate methods to assess the microbial community of anaerobic fermentation, which should help to increase the efficiency and ensure the stability of bio-methane production (Rozzi and Remigi 2004). The necessity for weekly analysis arises from the fact that weekly cost of this analysis is much smaller than the cost of revitalisation of the bioprocess keeled over in large scale plants. According to that need, three types of analyses applied in practice can be categorised as follows: molecular-, chemical- and microbiological-based methods. With the help of modern molecular fingerprinting techniques, it is possible to identify the microbial community involved in bio-methane process at the level of species. This enables researchers to see an exact spectrum of species at the DNA level, by which the identification of microbes becomes possible. However, this is time-consuming, expensive and not necessarily guaranteed to properly provide an actual status of the existing problem in the bioprocess (Hargreaves et al. 2013). The fingerprinting analysis is usually based on all DNA fragments in the sample which are obtained by the DNA extraction process, which cannot differentiate between lysed dead cells and living cells. Hence, it may not indicate the actual status. Another potential disadvantage of molecular-based methods is that they do not always generate absolute counts of active cells (Case et al. 2007; Větrovský and Baldrian 2013), which have traditionally served as an indicator for the activity of the bioprocess (Raskin et al. 1994; Elias et al. 1999; Kubota et al. 2009). In particular, a stakeholder in practice often requires qualitative and quantitative analysis of the biological status of fermentors based on rapid, inexpensive and stable techniques as a quick response to the operational problems. Regarding that demand of the stakeholders, numerous chemical analysis methods have been developed during the last decade (Scherer 2007; DBFZ, 2012). Those methods have fulfilled the demand for speed, but were not always satisfactory because they left many uncertainties around the complicated biological process while partially providing information about the number of chemical states. Thus, the bio-methane process has often been referred to as a 'black box' (Riviére et al. 2009; Kothari et al. 2014). Therefore, there is still a need to find a quick and more appropriate method, which has led to the

consideration of microbiological solutions. The urgent demand for analytical results to provide reliable microbiological solutions has recently soared to be able to understand the bioprocess in anaerobic digesters (Scherer 2007; Scherer et al. 2012). To determine the activity and progress of a bioprocess, cell quantification has been widely used in many areas of life science. Although there are already a lot of microbiological quantification methods, it has not been easy to apply the existing methods to an environmental sample of a bio-methane process due to the complexity of the matrix. A cell counting method for environmental samples using microscopy could be found in soil science by using fluorescence *in situ* hybridisation (FISH) which enables cell quantification, in some cases down to the level of the species (Daims et al. 2001; Krakat et al. 2010). However, the molecular-based FISH technique cannot be applied in our case, because it generally requires cell fixation by applying formaldehyde during the preparation of samples to prevent cell autolysis. Since the methanogenic activity of living cells is our research interest, the fixation step killing the cells could not be applied. Additionally, the environmental samples contain a large number of different chemical precipitates, particles, and plant fibres, which can disturb accurate estimations. Thus, there is a substantial need for optimisation of the quantification process. Quantification of microbial communities in anaerobic digesters is required for providing crucial information about the activity and progress of the microorganisms, which can be directly related to the bio-methane output (Kubota et al. 2009: Munk et al. 2010; Traversi et al. 2012). In this study, a microbial quantification method by microscopic image analysis was developed to monitor the stability of the bio-methane process in a practical context as well as furthermore to improve the process efficiency of digestion.

The driving force of the whole bio-methane process is methane-producing microorganisms among many other participating bacteria, so-called methanogens. Methane formation is a common phenomenon and has widespread occurrence in nature, for example in some digestion processes of organic matter in sludge, rumen and intestinal tracts of animals, as well as in the sediment and mud of aquatic habitats. Numerous studies on methanogens have been performed since the beginning of the 20th century (Barker 1936; Zeikus 1977; Jones et al. 1987; Thauer et al. 2008); the methanogens are, however, very interestingly, a under-investigated group due to their strictly anaerobic characteristics and ubiquitous syntrophic properties (Zeikus 1977). Consequently, the cultivation and isolation of methanogens has often failed. Despite those many failures in the isolation of methanogens, scientific interest and practical importance for methanogens

in the sense of purely scientific interest as well as of a carrier of sustainable energy production has increased (Costa and Leigh 2004; Christy et al. 2014). There have been several cultivation and isolation techniques introduced to date (Wolfe 2011). One emphasis of this present research lies on seeking out a simple, inexpensive, and universal method for the cultivation and isolation of H_2-CO_2-consuming methanogens in particular, which should contribute to developing and disseminating knowledge of methanogens. The H_2-CO_2-consuming methanogens, i.e. hydrogenotrophic methanogens, were found to be dominant in German farmer bio-methane plants, while acetate-consuming methanogens, i.e. aceticlastic methanogens, were often observed mainly in sewage sludge plants (Demirel and Scherer 2008; Demirel 2014). The farmer bio-methane plants have been drastically increased as an alternative source of renewable energy in Germany since 2000 due to application of the renewable energy law (EEG). Methanogens, however, were only specifically investigated, related to many still undefined species (Wirth et al. 2012). To cultivate methanogens, their general ecological information is fundamentally important. The methanogens enable the natural carbon cycle, usually at the lowest redox potential occurring in nature, where extreme conditions govern, like the absence of oxygen and light. Often, toxic and odoriferous hydrogen sulphide and ammonia are present as the degradation products of proteins. At the end of the food cycle, methanogens are able to consume only a few chemical compounds as substrate such as formate, acetate, methanol, hydrogen and carbon dioxide, which means they rely on leftovers from other bacteria for their growth and maintenance (Stams 1994). Many can only survive in a syntrophic context: e.g. they receive hydrogen directly through interspecies for energy, and conversely induce a thermodynamically more comfortable condition for the partner bacteria by removing the unfavourable product of these bacteria like hydrogen (Traore et al. 1983; Schink and Stams 2013). In that sense, methanogens have a role like sweepers. Interestingly, some methanogens could even grow by obtaining energy through direct electron transfer from partner microorganisms or atoms like iron, or from external electricity sources, i.e. electromethanogenesis (Daniels et al., 1987; Stams et al., 2006; Cheng et al., 2009; Lovely and Nevin, 2013, Rotaru et al., 2014). Even though methanogens can grow in such extremely odd conditions, they are ironically known as one of the most distributed and the oldest living organisms (Ueno et al. 2006), having survived even in hyper-thermal conditions such as 100°C. In the dark, methanogens uniquely shine in light blue and green, among many bacteria, by reflecting specific light with a wavelength at 420 nm, based on their characteristic coenzyme F_{420} (Cheeseman et al. 1972). In this way, methanogens can be found and distinguished very easily from concomitant bacteria under a fluorescence

microscope. Through methanogenesis, meaning the generation of methane, methanogens usually gain the energy to reproduce themselves, in other words, to build ATP for biosyhthesis. Methanogens use about 3% of carbon sources obtained for biomass growth; 97% of carbon flows into the formation of methane (Avaisidis and Wandrey 1985). In this study, especially in the context of an energy carrier to generate bio-methane, an optimised cultivation and isolation system for methanogens from anaerobic farmer digesters was developed to contribute to knowledge of methanogens. Furthermore, the isolates obtained by this developed technique were provided to partner institutes for subsequent molecular analysis, like genome sequencing, in order to establish the microbial profile of anaerobic digesters in Germany. This comprehensive molecular data of anaerobic digesters will help to open up the black box of farmer bio-methane plants at the genome level.

To summarise, the present research topic was developed from two arising demands: firstly, for a quick and simple microbial quantification method to analyse the activity of a bioprocess, and secondly, for a simple isolation method to be used for genome sequencing to contribute to the establishment of genome data for anaerobic digesters. The seemingly different parts combined as a single research topic aims at one goal: to unveil the biological black box of anaerobic digesters. The former is a quick method for the practical appliance at the micro-level and the latter is a simple method for the long-term, scientific application at the nano-level. The two parts will supplement each other and consequently facilitate the realisation of a more effective goal.

1.1.   Definition of research questions

Procedure for the development of methods to reach the research goal

The first part of the research topic regards the search for the best solution for the general quantification of microbes in anaerobic digesters, consideration of the development of proper methods, and the possibility of integrating the findings into an application in the field (figure 1).

Definition of Research Problem 1: Microbiological Approach for Quantification

Appropriate microbiological quantification method?	Counting chamber	- Microscope is availaible in all microbiological labs
	Plating and CFU counting	- Rapid (automatic counting high cell number 10^{11}/ml)
	Spectrophotometry	- Automatic morphological classification (unique method to see authentic bacteria)
	Microscopical image analysis	

New Research Problems: Establishment of Microscopic Quantification

Establishment of microscopy procedures for quantification of environmental sample?	3D → 2D homogenous in mono layer	- Appropriate pretreatment technique
	Estimation sample area	- Automated microscope
	Differentiation biotic target particles	- Staining organics and autofluorescence
	Establishment macros programming	- Programming and optimizing image quality - Validation with references

New Research Problems: Application of Microscopic Quantification

Application of the microscope technique	Numerous analyses, data collection	- Analysis of many samples of various digesters
	Search for new relationships	- Comparison of analysis data and operational parameters
	Generalising new findings, formula	- Development of a mathematical formular
	Proposing the findings	- Validation with other existing methods

Figure 1 Definition of research questions about microbiological approach for quantification of microorganisms in environmental samples

Through a review of the current literature on the topic, microscopic image analysis was found to be a proper microbiological quantification method for our purpose. By using a counting chamber as well as spectrometry, it was not easy to differentiate between cells and soil particles in the environmental samples. Plating and colony forming units (CFU) is a time-consuming cultivation-dependent method and concerns only active cells that can grow on the agar plate used, which is not an ideal method for the quantification of total cells. For successful application of microscopic image analysis, the following requirements had to be fulfilled: three-dimensional samples had to be converted to two-dimensional samples, and had to be homogenously distributed in a monolayer on a microscope slide. The distributed sample area had to be measured to finally calculate the

number of cells. Abiotic non-target particles had to be sorted. A macro programming had to be optimised, e.g. to minimise background fluorescence (see section 1.3.), to enhance image quality, and to optimise quantifying and classifying strategies.

The second part of the research topic involved the search for an optimum solution to the cultivation and isolation of hydrogenotrophic methanogens from German farmer anaerobic digesters, consideration of the development of appropriate methods, and the possibility of converting the findings into an application of the scientific field (figure 2).
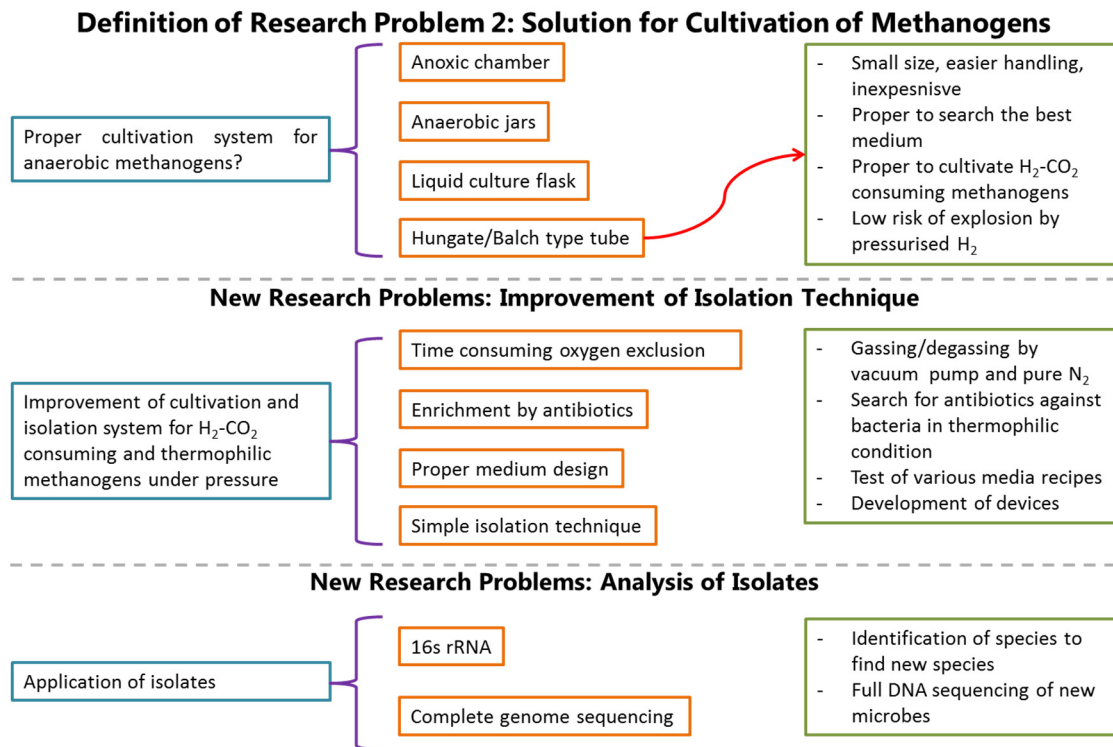
Figure 2 Definition of research questions about a cultivation and isolation method for H_2-CO_2-consuming methanogens

During a literature review on possible cultivation methods of anaerobic methanogens, some existing anaerobic techniques were identified (Balch and Wolfe 1976). To work under anoxic conditions, an anoxic cabinet can be used, but this is expensive, costly in terms of energy consumption, and has a space requirement. Anaerobic jars or 0.5-1 L liquid cultivation flasks can be used, but it is not appropriate for cultivating hydrogenotrophic methanogens as target microbes, as H_2-CO_2 as the energy and carbon source must be regularly pressurised to 2 bars of overpressure. Thus, a Hungate/Balch type tube was chosen as a simple cultivation vial, which is appropriate for our purposes, and has already been established in our laboratory (see section 2.2.2.).

However, some parts of the Hungate/Balch technique were modified in this study, according to the established methods of the laboratory. First, the anaerobic chamber and anaerobic jars could be omitted. The boiling of medium for the exclusion of oxygen, which is time-consuming, was replaced by a gassing/degassing step with the help of a vacuum pump and pure N_2, without an oxygen scrubber. For this, a metallic gas-tight manifold was assembled instead of a glass manifold in order to distribute gas to 12 tubes simultaneously under overpressure. The Hungate roll-tube technique (Hungate 1969) was applied in a 10 mL serum flask, where only the inner wall of medium was coated with an inoculated agar medium to enable more gas space in the flask and to be able to pick a colony much easier. Additionally, an anaerobic petri-dish box was developed in this study. This is a single plate system, which is pressure safe, and thermo-stable. Regular gas feeding is also possible. Through the use of a transparent cover, the colonies are detectable, meaning that opening the cover for every check is unnecessary.

The isolates were sent to partner scientists to be analysed using molecular methods like 16s rRNA and genome sequencing. The results were used for the establishment of microbial profiles of anaerobic farmer digesters in Germany.

1.2.   Research goal and scope

The research goal was to develop a practically reliable analysis method for the microbial quantification of anaerobic digesters, facilitating improvements of the efficiency of bio-methane production. Another important research goal was the development of a simple isolation process for methanogens with respect to gas pressurisable anaerobic vials as a consequence of optimised cultivation techniques (figure 3). At the intersection point of both parts of this study, it is expected to provide new knowledge and an improved, new tool to analyse microbial activity and diversity in anaerobic digesters. The research scope will focus on the optimisation of microscopic techniques and their application in practice, as well as optimisation of cultivation and isolation techniques.
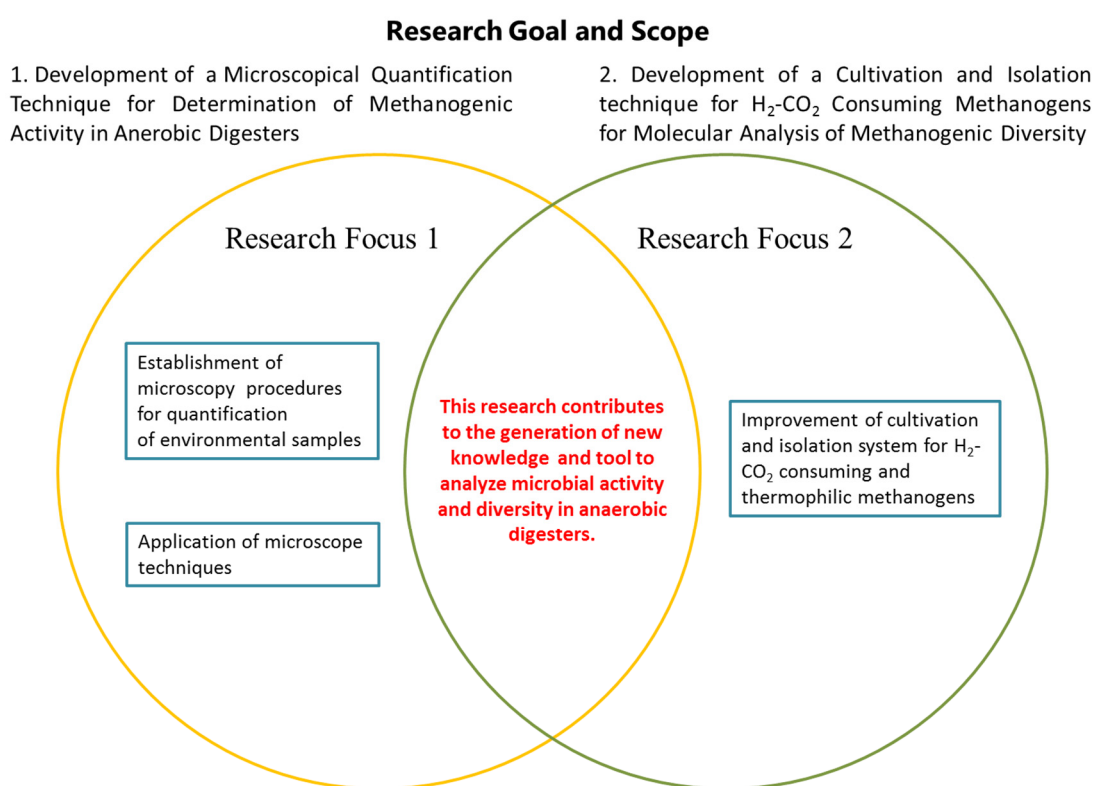
Figure 3 Research goal and scope

1.3. Literature review for development of the used microscopic technique

Among the several types of analysis in microbiology, only microscopic methods provide a direct image of bacterial cells. Determination of absolute living cell number (N/mL) of an environmental sample allows a direct indication of the cell activity throughout cell growth, which enables a prognostic calculation in the anaerobic digestion simulation. A number of microscopic count methods have been developed since the middle of the 20th century (Utermöhl 1958; Zimmermann et al. 1978; Zweifel and Hagström 1995; Culverhouse et al. 1996; Blackburn et al. 1998; Singleton et al. 2001; Pernthaler et al. 2003; Zeder et al. 2010). Initially, determination of cell abundance by microscopy was routinely performed by manual counting, but semiautomatic and automatic counts with image analysis have been implemented over time. Accordingly, the quantification of cells using fluorescent stains has been of interest for over 35 years (Zimmermann 1975; Hobbie et al. 1977). Since environmental samples contain high amounts of other particles like soil particles and plant debris, as well as unknown, possibly uncultivable species, some counting methods such as counting on agar plates or optical density (OD) were not taken into account in this study. In most methods used to quantify total cells by microscopy, (para) formaldehyde has been used to fix the cells before staining or hybridisation (Amann et al. 1990; Kepner and Pratt 1994; Weinbauer et al. 1998). In our case, we omitted this fixing step with paraformaldehyde because methanogens lose auto-fluorescence and vitality through this fixing process. The membrane filter technique has widely been accepted in the fluorescence *in situ* hybridisation (FISH) technique. Staining of cells was necessary to differentiate between biotic particles and abiotic particles because dye normally binds to DNA or RNA of organic samples. Although Francisco et al. (1973) reported that the introduction of black-stained membrane filters overcame some of the problems associated with background fluorescence, the problem of background fluorescence was still not solved (Weinbauer et al. 1998). Bloem (1995) tried to count cells directly on the microscope slide after drying the sample (meaning dead cells), without a membrane filtration step, and reported less background fluorescence and less fading of the fluorochromes in smears than on the membrane filter. Unfortunately, this method was less common in research implementation in contrast to membrane filter techniques for unknown reasons, probably due to the difficulty of initiating the homogenous distribution of objects directly on the slide. Unspecific fluorescence from the soil particles, which may exhibit higher fluorescence than target cells, was also still a

significant problem (Kuwae and Hosokawa 1999). In our experience, this problem might be reduced to some extent by dilution steps. Generally, membrane filtration has been applied coupled with cell fixation by paraformaldehyde to fix the cells. Instead, methylcellulose with low viscosity was used in this study to coat the microscope slide to absorb the surplus water of the sample and fix the cells in a monolayer (modified from, Bast 1999). One advantage of this method was that the auto-fluorescence activity of methanogens can still be estimated.

A variety of approaches to quantify cells currently exist. They can be categorised into indirect and direct quantification or absolute and relative quantification of cells. Indirect quantification has been based on several methods such as ether-linked glycerolipids (Ohtsubo et al. 1993), coenzyme M (Elias et al. 1999), fluorescence intensity (Xue et al. 2009), DNA extraction (Aoshima et al. 2006), gene copy number of quantitative PCR (Steinberg and Regan 2009), or denaturing gradient gel electrophoresis (DGGE) (O'Reilly et al. 2009). The above methods did not become standards for quantification, with the exception of qPCR. However, qPCR was more closely related to the qualitative analysis of cells due to unknown factors like different gene number and sample-specific inhibition during quantification (Case et al. 2007; Hargreaves et al. 2013). Direct quantification was carried out using FISH (Kämpfer et al. 1996; Daims et al. 2001; Ravenschlag at al. 2001), or fluorescent staining methods (Kepner and Pratt 1994; Weinbauer et al. 1998; Klauth et al. 2004). This absolute counting is generally performed by manual counting in FISH studies; for instance, the number of stained target cells can be obtained as a percentage of the total cell count (Delong et al. 1999).

Several stains for the quantification of total cells have been suggested such as acridine orange (AO), 4', 6-diamidino-2-phenylindole (DAPI), SYBR Green or Sytox Green (König et al. 2000), in order to differentiate between organics and non-organics. Fluorescent stains can be divided into cationic (e.g. AO, DAPI) and anionic stains (e.g. fluorescein isothiocyanate (FITC), 5-4, 6,-dichlorotriazin-2-aminofluorescein (DTAF)). AO intercalates DNA and RNA, as does DAPI. However, AO also binds other negatively charged cellular constituents, detritus and clay meaning that AO exhibits more non-specific background staining. The pH value and salt concentration may be influencing factors for staining with fluorescence dyes (Bölter et al. 2002). Bitton (1993) found that the fluorescent cells stained with AO were influenced by the level of moisture on the filter. Therefore, AO seemed to be an unsuitable stain. Problems with DAPI have often been reported as well, as not all cells are stained with DAPI or a very low staining grade was

reached in living cells (Weinbauer et al. 1998; Klauth et al. 2004). Martens-Habbena and Sass (2006) evaluated AO, DAPI, ethidium bromide, PicoGreen, and SYBR Green I and II. They found that SYBR Green I was the most sensitive dye for the determination of cell numbers. Furthermore, dyes of the SYBR-type were less toxic and did not reveal any mutagenic properties (Singer et al. 1999; Kirsanov et al. 2010). Kepner and Pratt (1994) reported that different standard procedures for staining can cause different results between AO and DAPI. Standardisation of the pre-treatment procedure seemed to be a significant factor. Klauth (2004) reported on another fluorescent dye, Sytox Green, which has shown to have more specific properties than SYBR Green I. However, it showed a recovery rate of 81% in the spiked cells that were stained by Sytox. On the other hand, Klauth (2004) suggested a procedure to recognise cells on the soil particles separately by the fluorescent enhancement of a DNA-bound dye and sufficient spectral differentiation with Sytox Green. Unfortunately, however, this dye is not permeable to living cells (Mason et al. 1998; Klauth et al. 2004). That is perhaps the reason why Sytox Green has been used much more commonly as an indicator for the discrimination of dead cells from active cells, rather than for the quantification of total cells. To the best of our knowledge, this staining with Sytox seemed to be more complicated due to the additional step required for spectral differentiation between soil particles and bacteria. For the reasons stated above, in addition to our experiences, SYBR Green I was chosen as a stain in this study. Another method for the quantification of cells is flow cytometric analysis, which can measure cell size, DNA- and rRNA content of the fluorescein-labelled probes hybridised samples. However, this method also showed problems when counting aggregated and attached cells on soil particles (Wallner et al. 1995).

Phase contrast microscopy can be used to count mono-cultures or relatively simply structured samples, but it is not appropriate for counting total cells of environmental samples due to interfering particles. For counting methanogens, their auto-fluorescence could be used successfully. It is well known that the auto-fluorescence of methanogens is invoked by the characteristic factor F_{420}, which occurs abundantly in methanogens (Cheeseman et al. 1972; Edwards and Mcbride 1975).

Since microscopic manual counting of cells is normally a time-consuming, error prone process, number of automatic methods have been proposed (David and Paul 1989; Blatt et al. 2004; Russel et al. 2009) which can circumvent user-dependent errors (Bloem et al. 1995b). However, some problems like indistinct and irregular cells or aggregated cells in flocs and biofilms or soil particles in environmental samples still remain, meaning that it

is difficult to find an adequate threshold of intensity between background and target cells; in other words the segmentation of the target object from the background. This phenomenon still hampered application of the computational tools (Bloem et al. 1995b; Daims et al. 2001; Blatt et al. 2004; Russel at al. 2009). Nunan (2001) reported that the morphological features of cells are often similar in shape and size to the nonbacterial particles. Maniloff (1997) reported that the minimum size of bacteria may be 0.14 μm, which is similar to the size of soil particles. In particular, the separation of cells from soil particles has been reported to be impossible by several authors (Kepner and Pratt 1994; Kuwae and Hosokawa 1999) in phase contrast mode.

A simple semi-automated quantification method was developed, which is totally based on microscopic image analysis without any uncertain hybridisation with a gene probe and membrane filtration step. Furthermore, this rapid method provides a fingerprint of cellular morphotypes. For the monitoring of anaerobic digesters, the information of cell counts as well as morphological changes may be a new operational indicator, improving the bioprocess. For instance, aceticlastic methanogens and hydrogenotrophic methanogens can be distinguished morphologically as well as coccoid-type, rod-type, and filament-type within hydrogenotrophs (Kim et al. 2014).

In this study some experiments were carried out for the optimisation and validation of this technique. This microscopic technique was applied in many lab-scale and large-scale biogas plants to identify a new relationship between microscopic data and operational parameters. Furthermore, a microscopic index was developed for the rapid analysis of microbial quality in anaerobic digesters.

1.4. Literature review for development of an isolation technique for methanogens

In 1936 (Barker), the known methane-producing bacteria were summarised, which had been cultivated and characterised. It was well known that little success for isolation of methanogens often occurred by ordinary methods due to their strictly anaerobic characteristics and ubiquitous symbiotic properties. Although methane formation is a common phenomenon and widespread in nature, such as in digestion processes of organic matter in sludge, rumen and the intestinal tract of animals, in the sediment and muds of aquatic habitats, this group of methane-producing bacteria is still poorly known, i.e. there are still many unknown and uncharacterised methanogens (Sakai et al. 2007; Parkes et al. 2010; Wirth et al. 2012). Thereafter, a more precise study on methanogenic bacteria was reported by Zeikus being a scholar of Wolfe in 1977, especially in terms of biochemical characteristics and physiology (Zeikus 1977). Afterwards some comprehensive, general reviews on anaerobic cultivation techniques followed in 1986 (Ljungdahl and Wiegel), a very good overview of various anaerobic cultivation systems in 1989 (Fung), as well as some important remarks on the anaerobic technique in 2005 (Plugge). Plugge mentioned that two crucial points for the growth of anoxic prokaryotes were the exclusion of oxygen and the choice of appropriate growth media. Recently, a review on the anaerobic cultivation technique was published by Wolfe (2011), who already introduced the same topic in 1971. Although techniques for cultivating methanogens vary in detail among laboratories, the most widely used methods are based on the work of a pioneer in anaerobic techniques, Hungate (1950, 1969), which perfected the anaerobic medium preparation technique. Wolfe summed up the main points of Hungate technique in three parts: the exclusion of oxygen in the preparation and sterilisation of anoxic media, methods for the aseptic inoculation and transfer of anaerobic microbes in media where redox potential was maintained below -330mV. In the history of cultivation and isolation of methanogens, an innovative technique was developed which opened a new paradigm in the cultivation of methanogens, i.e. cultivation in a pressurised atmosphere (Balch and Wolfe 1976), facilitating the reduction of possible contamination and prohibiting the loss of reducing potential.

First of all, suitable vials for culturing strict anaerobes must be chosen which ensure anaerobic conditions and simple handling for sampling, inoculating, and feeding, as well as gassing in the case of pressurised cultivation. Various tubes, dishes, flasks, jars or glove boxes have been tested for anaerobic cultivation and isolation to date (Fung 1989). The

most commonly used and suitable types of vials are Hungate-type (Hungate 1969) and Balch-type (Balch and Wolfe 1976) tubes, which enable completely gas-tight closures. The Hungate-type tube was little modified with time and was closed with a screw cap and butyl rubber septum. The Balch-type tube has a butyl rubber stopper and aluminium crimp seal, which enables gas tightness under an overpressure of 2 to 3 bar during cultivation, meaning that it is more stable. Serum bottles with a butyl rubber stopper and aluminium crimp seal in various sizes were also used (Miller and Wolin 1974). Of particular note was that significant amounts of gas can be produced in a larger cultivation medium. Thus, the smaller the size of the vials, the easier they were to handle. The liquid medium should fill only 25-30% of the vials due to gas storage, in the case of H_2-CO_2-consuming methanogens. The material of the rubber stopper was of crucial importance. The suitability of butyl rubber stoppers was proven to be efficient for prevention of the permeation of air into the vial (Hungate 1966). However, the repeated puncturing of black butyl stoppers with injection needles made them permeable. There is a new type of synthetic blue butyl rubber stopper (DUNN, Asbach, Germany, CLS-4209-14) which has essentially shown more stability. Neoprene was also reported to be more stable than butyl rubber stoppers (Daniels and Zeikus 1975).

Oxygen-sensitivity of methanogens was investigated in 1983, as methanogenesis was inhibited by dissolved oxygen concentrations below 30nM (Scott et al. 1983). The exposure to air led to the death of methanogens on the solid medium within 10-30h depending on species, i.e. viability of *Methanobacterium* could last up to 30h whereas *Methanococcus* was killed within 10h (Kiener and Leisinger 1983). The solubility of oxygen in the air under 1 bar is 8 mg/L (250 nmol O_2 in water) at 25°C or 4.6 mg/L (144 nmol) at 60°C, which means that it is difficult to create anaerobic conditions. To remove oxygen during media preparation and cultivation, a vertical column of electrically heated (350°C) copper scrubber can be used to purify the used inert gases (Hungate 1969). However, an easier method for removing oxygen seemed to be the method involving boiling medium with a vacuum pump and the addition of reducing agents including cysteine, sodium sulphide as reviewed by Wolfe (Wolfe 2011) and Titanium (III) citrate (Zehnder and Wuhrmann 1976; Jones and Pickard 1980; Wachenheim and Hespell 1984). The redox potential of sodium sulphide, Titanium (III) citrate, and cysteine hydrochloride in standard conditions is -517, -480, and -210 mV, respectively (Ljungdahl and Wiegel 1986). Titanium citrate can be used as a reducing agent but can inhibit strict anaerobes depending on the growth rate and its concentration (Wachenheim and Hespell 1984),

which is in contrast to non-toxic cysteine (Zhang and Maekawa 1996). In general, resazurin is supplemented as a simple and excellent redox potential indicator which is reddish when oxidised and colourless when reduced at -330mV (Wolfe 2011).

The choice of medium components is one of the most crucial factors for the successful cultivation of methanogens. The characterisation of microorganisms isolated in an artificial defined medium is necessary but it can very often lead to time-consuming work or no cultivation success due to the lack of specific nutrients required for growth. A too high concentration of the substrate can also inhibit the growth. In most cases of cultivation, a high hydrogen concentration (1–2 bar) was used. On the other hand, an interesting approach was conducted to keep a low concentration of hydrogen about at $1 - 100$ Pa, which is actually similar to conditions of methanogenic natural habitats in order to isolate novel methanogens; such a low concentration of hydrogen was achieved specifically by co-culture (Sakai et al. 2007). However, in practice, it is technically very difficult to create and maintain a low concentration of hydrogen. In order to create the growth condition like natural habitats of methanogens, the application of relatively low concentration of nutrients, non-traditional sources of nutrients and relatively lengthy periods of incubation were suggested for the successful novel developments to culture the uncultured microbes (Plugge, 2005). The conventional, complex media for methanogens contained carbon sources like CO_2, formate, methanol, sodium acetate, some macroelements like K, Mg, S, Na, Cl, Mg, Fe (Balch, 1976), micronutrient solutions and vitamin solutions (Wolin 1963) including undefined growth stimulators such as clarified rumen fluid, kelp digester supernatant, yeast extracts, and trypticase. Among these stimulators, rumen fluid and trypticase were reported to be the additives (Ferguson and Mah 1983) causing the highest growth rate. Another essential point was the pH controlling of the medium by buffer system, e.g. bicarbonate or carbonate (Wolfe, 2011), especially when regularly feeding CO_2 as a carbon source for the cultivation of hydrogenotrophic methanogens. The fermentor medium (natural medium) from the initial sampling habitats can reduce incubation time (adaptation time).

Several attempts to develop proper cultivation systems, especially for H_2 and CO_2-consuming methanogens, were performed besides the basic batch system using the roll-tubes of Hungate/Balch (Hungate 1969; Balch and Wolfe 1976). In 1966, a simple modified anaerobic jar for solid culture plates was constructed consisting of an anaerobic lid with a snap-in rubber gasket, palladium pellet catalyst to activate hydrogen generation from a disposable hydrogen-carbon dioxide generator, and a methylene blue anaerobic

indicator inside the jar (Brewer and Allgeier 1966). Two years later, a method for the liquid mass culture of hydrogen-oxidising methanogens using a 200 mL shake flask and 12 L fermentor with apparatus for providing a gas mixture of 80% H_2 and 20% CO_2 was introduced and successfully employed for the mass production of cells (Bryant at al. 1968). Afterwards, a slightly improved liquid culture system was introduced (Daniels and Zeikus 1975). The gas inlet and outlet were equipped for continuous gassing and with a side arm designed to allow turbidity measurements of cells, as well as a neoprene-stoppered inlet, which can be conveniently changed. The solubility of hydrogen is extremely low, with a level of 0.0014 g H_2/kg H_2O at 37°C (about 1000 times lower than CO_2), which means that the overpressure of hydrogen was necessary. However, the overpressure of hydrogen was impossible in such a system. Since the aim of this study involves a simple method for the cultivation and isolation of methanogens, the modified Hungate type roll-tube was used as a semi continuous culture system for hydrogen oxidising methanogens.

The difficulty in isolating methanogens on solid media has been very well known, probably due to O_2 inhibition (Barker 1934, Jones et al. 1983). Traditionally, the roll-tube technique (Hungate 1969) was used for isolation, in which agar was spread evenly over the inner of surface. The inside of the tube is empty to make picking colonies easier. The bacterial culture was imbedded or streaked along the inner side of the agar by turning the roll-tube with a streaker. The colonies can be picked by touching with an inoculating needle (Hungate 1969, Wolfe 1971). In addition, an interesting technique for the isolation of methanogens was reported (Edwards and Mcbride 1975) by using an anaerobic chamber in which petri-plates were placed. For the simple detection of methanogenic colonies, the auto-fluorescence of coenzyme F_{420} was used under ultraviolet light, e.g. of LED flashlight. Cultured blue-green fluorescent colonies were not always pure but they were all methanogens. More convenience for picking the colonies was found in the petri-dish technique than in roll-tubes in terms of easy access to the colonies. However, the petri-dish technique needs either an anaerobic chamber or a pressurised box to establish the necessary anoxic environment which represents a considerable investment or explosion risk. Another isolation technique was found which did not need anaerobic chamber, i.e. agar bottle plate. The agar bottle plate provided the convenience of an agar streak plate, solved the problem of the water exudate from the agar medium and facilitated an easy way of adding and sampling a defined gas atmosphere (Hermann et al. 1986). However, it seemed to require much more H_2 and CO_2 to be in the glass bottle up to 1-2

bar for the cultivation of H_2-oxidising methanogens, which may be related to an explosion risk. A more sophisticated and direct picking technique using a micromanipulator was reported for prokaryotes under direct visual control (Fröhlich and König 2000). However, it is bound to a high investment cost and a risk of exposure to O_2 contact during the procedure. Thus, in this study, a simple modified and combined technique from Hungate, Balch, and Miller, i.e. 10ml serum bottle with butyl rubber stopper and aluminium crimp seal, was used for isolation as a simple method, with the same principle as a roll-tube (figure 11). This technique enabled easier picking colonies by cannula. Additionally, an anaerobic petri-dish box was developed in this study which took advantage of the petri-dish technique but without anaerobic chamber. A single petri-dish was put inside the box, which can be pressurised up to 2.5 bars (figure 12).

The present study was focused on the isolation of a novel hydrogenotrophic methanogen from mesophilic and especially thermophilic digesters (Ferguson and Mah 1983; Harris at al. 1984; Blotevogel et al. 1985; Zhao et al. 1986, Kamagata and Mikami 1991). With the help of the literature and a number of attempts by our group in the past (Scherer and Sahm 1981), a modified Hungate roll-tube and Balch-gassing manifold system for the simple cultivation, an optimised medium including reducing agents and a 10mL serum bottle and anaerobic petri-dish box for the isolation of methanogens were developed and employed in this study.

The materials and methods used for the cultivation and isolation of methanogens are described in the next section. Furthermore, some results of the optimisation procedures as well as some isolates from this study will be presented in the results section.

Chapter 2: Methods and Materials

We cannot solve our problems with the same thinking we used

when we created them

Albert Einstein

2. Methods and materials

2.1. Methods for microscopy

2.1.1. Pre-treatment and microscopy

Pre-treatment for microscopy

A mono-culture was normally undiluted or diluted twice with PBS (PBS, 10 mmol sodium phosphate buffer inclusive 130mM NaCl, pH 7.2). Environmental samples were diluted thirty or sixty times with PBS as a final concentration depending on sample consistency, to get individual cells and differentiate cells from the untargeted particles. About 5g of sample was taken in a closed cup without extra flushing with N_2. If the environmental sample contained a high solid content more than 3% total solid content, it was pre-diluted two (solid content < 6%) or three times (solid content > 6%). This solution was diluted ten times with anti-fading agent DAPCO-PBS solution (DABCO: 1, 4-Diaza-bicyclo (2,2,2) octane, Roth 0718, Karlsruhe, Germany: 5g DAPCO mixed with 100 mL PBS). Total cell counts and methanogenic cell counts were prepared differently. A 10 µL aliquot of the mixture was used for methanogenic cell counts. For total cell counts, a separate slide was prepared. Here, 5 µL of SYBR Green I was mixed with a 5 µL aliquot of the mixture on the microscope coverslip directly by pipetting for 10 seconds. SYBR Green I (Life Technologies Darmstadt, 10000x concentrate, S7563) working solution represents a thousand times dilution of the stock solution (10000x concentrate) (Weinbauer et al. 1998; Klauth et al. 2004). The final concentration of SYBR Green I was 9.8 µmol (Zipper at al. 2004). Since fluorescence is very sensitive to light, this indicator should be handled in the dark and stored covered with aluminium foil at 4°C. The working solution of SYBR Green had a shelf-life of approximately 1 month. The stock solution was stored at -20°C. Whole preparation and microscopy was carried out in the dark.

After 10 µL of the pre-treated sample was pipetted onto the coverslip (24 x 50 mm^2) as described before, one side of the coverslip with this 10 µL drop (sample side facing down) was placed on an angle on the methyl cellulose-coated microscope slide (400 mPas, figure 27) and the other side of the coverslip was dropped. Distribution of the liquid sample under the coverslip was achieved by dropping down the coverslip. Additional pressing of the coverslip caused non-homogenous distribution, i.e. an additional ring shaped distribution normally followed. As a result of dropping the coverslip, the sample was distributed rapidly and widely enough, i.e. the area of the distributed sample covered at

least 17% (200 mm^2) of the coverslip (24 x 50 mm^2). The whole sample had to be placed within the area of the coverslip to enable calculation of the area.

To coat the microscope slides with the coating agent of methyl cellulose, a metal holder and coating stick was developed in this study in order to minimise person-dependent deviations (figure 27). Microscopic slides, with a thickness of approximately 1 mm, were placed on the holder, with a maximum depth of 1.2 mm for the slide, leaving a space of 0.2 mm for the methyl cellulose. Therefore, 350 µl of methyl cellulose was pipetted onto the left corner of the microscope slide. It was mixed by pipette tip for 10 seconds and coated by moving the stick from the left corner to the right. After drying for 25–30 min at 50°C on a thermo-plate (OMNILAB, Germany), it was ready for use.

Microscopy

The microscope used was a Leica DM6000B with a motorised and PC-controlled three-axis cross table. The images were captured by a Leica DFC365FX camera controlled by the Image Pro 7 software (MediaCybernetics, Bethesda USA). The prepared slide was placed on the microscope table. The entire image was checked once at a magnification of 50X to ensure that the distribution was homogenous without any ring shape or dense area. The acquisition position was chosen in a corner of the sample area with a middle line and the images were captured while moving to another corner horizontally or vertically with a gap of an image size. The images should be as representative as possible. Focusing and acquisition of the images were performed manually.

For each sample, approximately 20 images were captured in succession in the chosen area at 400-fold magnification. For the quantification of total cells and methanogens, different fluorescence filter sets were used. A fluorescence filter set (Leica CFP, excitation 426-446 nm, emission 460-500 nm) was used for the detection of methanogens. A filter set (Leica L5) with an excitation of 460-500 nm and an emission of 512-542 nm was used for counting total cells stained with SYBR Green. Thereby, shutter speed and signal intensification of the picture should be regulated depending on the fluorescence of the sample and recorded, but it should be continuously kept and not changed for a sample series; in our case, 82.4 milliseconds and 4 times, respectively.

2.1.2. Automatic counting and classification

Cell counting and the discrimination of cell morphology could be automatically carried out by image Pro 7. Mathematical algorithms for image filtration based upon morphology particularly allowed distinguishing cells from the non-cellular particles. Simultaneously, algorithms for digital image filtration in terms of frequency, grey scale and discrimination of overlapped objects were adjusted. In the fluorescence mode, the High-Gaussian function filter with a matrix of 7x7 (2 passes, 10 strengths) was used for the enhancement of fine image details. A morphological open function filter with 2x2 squares was used to smooth object contours, to separate narrowly connected objects and to remove small dark holes. To flatten the background of the images, the flatten filtration function (feature size 10 in pixels) was applied. In each image, the intensity range of the grey scale was automatically selected by Image Pro 7 to avoid user-dependent errors.

To display the filtered and measured images, some options were applied as follows: Counted objects were highlighted with an electronically coloured trace around the perimeter with no label; Objects that intersected the designated edges of the image were also counted. Counts could be excluded from those objects that were embedded within other objects. The objects were generally smoothed with a smoothing value of 15 in the program.

To distinguish cells from non-cellular particles or plant fibres, some rules of size filtration were programmed, based on the work of former master student Y. Shaikh. The filtration rules were applied differently to count fluorescing methanogens and total cells (see macros used in appendix). For a mono-culture, relatively simple rules with length and light intensity were used as those particles with a length between 2 and 7 μm were counted. Using a function of mean density, for example, thereby targeted dark objects in a bright background could also be differentiated.

For an environmental sample, all objects with an area less than 0.1 μm^2 were not counted, along with objects larger than 300 μm and with an area bigger than 100 μm^2. Additionally, atypical cell shapes such as objects with a length of the minor axis of an ellipse more than 4 μm were excluded. Some objects that were very similar to coccoid cells but which were not cells (just bright spots in phase contrast) could be filtered by setting the density filtration at 100 (max. 255) in the phase contrast mode.

By using geometric filtration options, the counted cells could be classified into rod- and coccoid-type by using the function of aspect (ratio between major axis and minor axis of ellipse), representing coccoid cells with a ratio less than 1.5 and rods with a ratio of more than 1.5. In addition to this aspect, cell type could be further classified into long rods and short rods by the length.
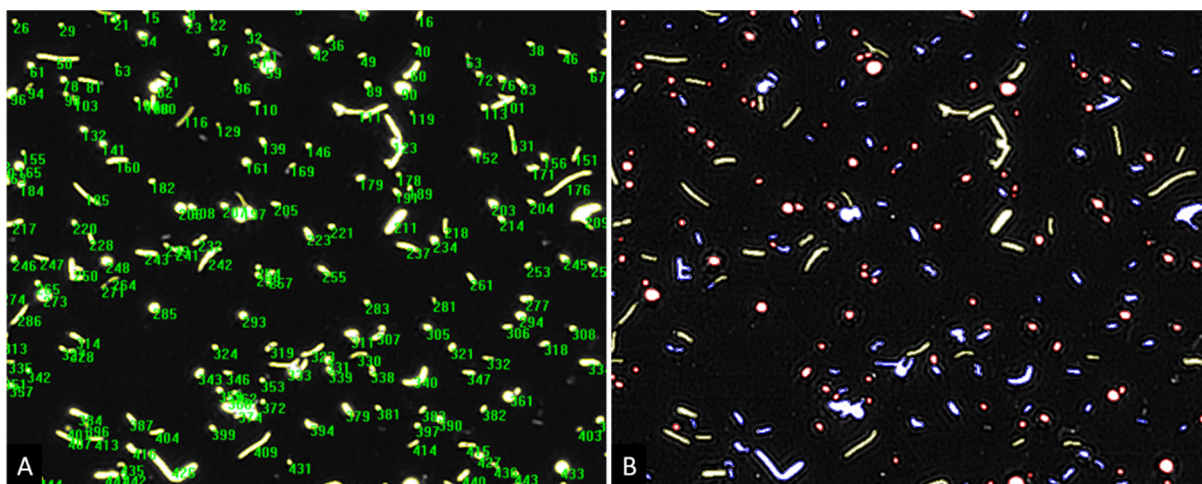
Figure 4 Example of automatic counting (A) and classification (B) by image analysis

Each cell received a number, and each object was measured and morphologically differentiated on the basis of different colours.
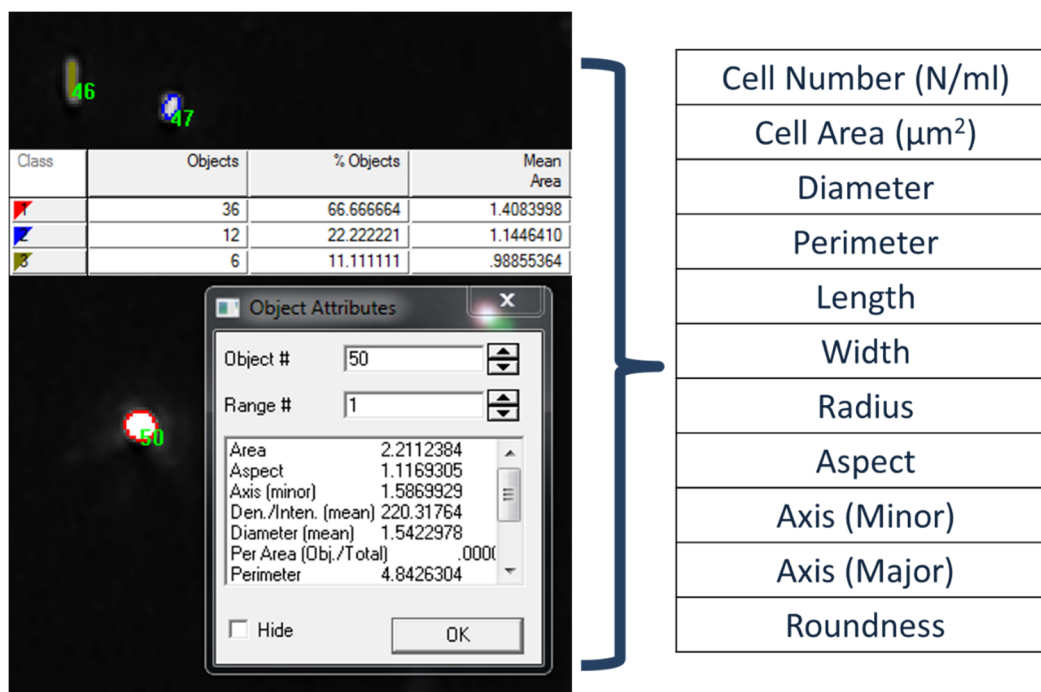
Figure 5 Automatic classification denoted with different colours and measurements

Every targeted object (e.g. object number 50) was measured using more than 50 parameters, including area, diameter, perimeter, length, radius, aspect, axis, roundness etc.

2.2. Methods for isolation of methanogens

2.2.1. Chemicals

For the isolation of methanogens, various media were tested and applied; e.g. several DSMZ media (141, 287, 318, 332, and 334) were applied as well as the medium "HAW", which was developed in our laboratory on the basis of Scherer (1989). The HAW medium was prepared at two different pH (pH 6.4 and pH 7) in order to separate methanogenic coccoids from methanogenic rods (Stantscheff et al. 2014). For the isolation of *Methanosarcina,* HAW-*Sarcina* medium was made which possesses the same components as the HAW medium (pH 6.4), but includes Methanol (150 mM) instead of other carbon and energy sources like acetate, formate, carbonate and hydrogen.

Table 1 HAW-medium (as developed on the basis of Scherer, 1989)

Nutrients							
Microelement (mM)		Trace element (µM)		Vitamin (µM)		Additives (mM)	
$MgCL_2*6H_2O$	5	$ZnCl_2$	0.5	Biotin	0.08	Imidazole	10
NaCl	30	$MnCl_2 * 4H_2O$	0.1	Folic acid	0.05	$(NaPO_3)x$	1
NH_4Cl	18.6	H_3BO_3	0.1	Pyridoxinhydrochlorid	0.49	$NaHCO_3$	5
$CaCl_2*2H_2O$	0.5	$CuCl_2 * 2H_2O$	0.1	Riboflavin	0.13	Sodium Acetate	5
KCl	64.1	$NaWO_4 * 2H_2O$	0.1	Thiamin (Vitamin B_1)	0.15	Sodium Formate	10
		$CoCl_2 * 6H_2O$	0.5	Nicotine acid	0.41	Resazurin	8µM
		$NiCl_2 *6H_2O$	0.5	Ca-Pantothenate	0.1	Cysteine	0.425
		$Na_2SeO_3 * 5H_2O$	0.1	Vitamin B_{12},	0.0015	Na_2S	1
		$Na_2MoO_4 * 2H_2O$	0.1	*p*-aminobenzoic acid	0.36	Titanium (III) citrate	0.083
		$Fe(III)Cl_3*6H_2O$	10	Lipoic acid	0.24		
		$(NaPO_3)x$	1000				

Yeast extract (Bacto[TM], extract of autolysed yeast cells, 212750, BD) and tryptone from casein (Bacto[TM], pancreatic digest of casein, 211705, BD, same as trypticate) were added (0.5%, w/w) for a solid medium.

The details of all chemicals used for this medium (Table 1) are summarised in the appendix. From all media, oxygen was excluded by boiling the medium with a vacuum pump and flushing with pure N2. All media were anaerobically stored with overpressure (0.5 bar) of N2. Depending on medium components regarding heat stability, the medium was sterilised by autoclave or filtration (0.25 µm pore size) (see appendix).

Sometimes, fermentor medium was used as an additive. Fermentor medium was centrifuged at 13000 g for 1 hour. The supernatant was diluted twice (1:2) with PBS. It was filtered by a syringe filter (pore size 0.25 µm) and stored anaerobically in a 10 mL serum flask (application 1:50).

Procedure of preparation for a 200 mL medium

A 186 mL macroelement solution was prepared in a 250 mL beaker. For this, 2 mL imidazole (1 M), 2 mL acetate solution (0.5 M), 2 mL formate solution (1 M), 2 mL trace element solution, 2 mL vitamin solution, 400 μL resazurin (4 mM), 100 μL polyphosphate (2 M), and 1mL $NaHCO_3$ solution (1 M) were given to the macroelement solution. The pH value was adjusted with HCl (1 M) to pH 7. This solution was sterilised by filtration (0.25 μm pore size) with a vacuum pump. The solution was transferred to a sterile 200 mL serum flask in a sterile bank. The flask was closed with a butyl rubber stopper including aluminium caps. Oxygen was excluded by boiling the medium with a vacuum pump and aeration with pure N_2 several times (8–10) during strong mixing with a magnet stirrer. Then, 400 μL cysteine solution (212.5 mM), 800 μL sodium sulphide (250 mM), 200 μL titan citrate solution (83.9 mM) were added to the anaerobic solution. The pH was adjusted with HCl at 6.4 or 7.0 depending on the expected isolate. An overpressure of pure nitrogen of 0.25 bar was placed in the serum flask for storage under anaerobic conditions and to prevent the introduction of air to the flask.

2.2.2. Anaerobic technique

There are several essential conditions for cultivating methanogens. First of all, anoxic conditions must be made by boiling the medium at room temperature under vacuum and by flushing with pure N_2 (quality 5.0, 99.995%, Air liquide, Stelle, Germany) to remove oxygen from the medium. Additionally, the medium has to be reduced by cysteine and sodium sulphide as reducing agents to ensure that the reduction potential must be below − 330 mV. Secondly, components of the medium must contain all of the necessary nutrients in appropriate amounts for the growth of methanogens. The original salt concentrations (Scherer 1989) were reduced to avoid precipitation. It is also important to add the nutrients regularly afterwards, especially trace elements. For buffering medium, imidazole-including carbonate buffer was used. Phosphate buffer should be avoided because of precipitation. Thirdly, a simple measurement of the growth whilst under anoxic conditions has to be established to determine which medium is optimal for the growth of methanogens or to assess the growth. To measure the change of growth an electronic manometer (manufactured by SI special instruments, Nördlingen, Germany, product number 1908 for the HAW) was assembled, which is equipped with a male Luer-lock fitting in the front part of the pressure sensor to make leak-free connections with

commercially available needles (Scherer 1990). A fluorescence microscope was also used to identify the methanogens based on the fact that only the living methanogens under anaerobic conditions express blue-green auto-fluorescence from the unique methanogenic coenzyme F_{420} (Cheeseman et al. 1972; Edwards and Mcbride 1975; Gorris and van der Drift 1994).
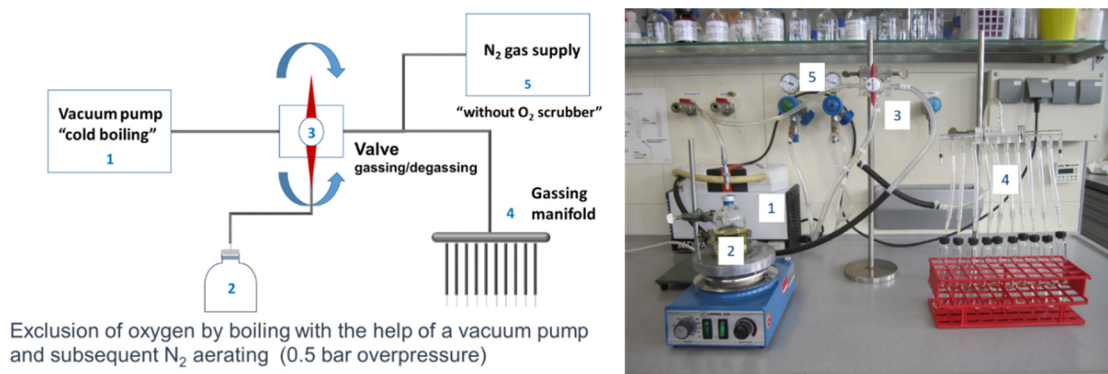
Figure 6 Exclusion of oxygen by gassing and degassing with a vacuum pump and pure N2 (99.995%)

Exclusion of oxygen from all procedures and media is essential. This step was simplified and accelerated by a vacuum pump and pure nitrogen gas, since the traditional method 'boiling by heat' takes a lot of time, especially the cooling step for the medium. During strong mixing of the medium with a magnetic stirrer, the boiling by vacuum pump and aerating with N_2 were repeated several times (8-10).

Cultivations vials
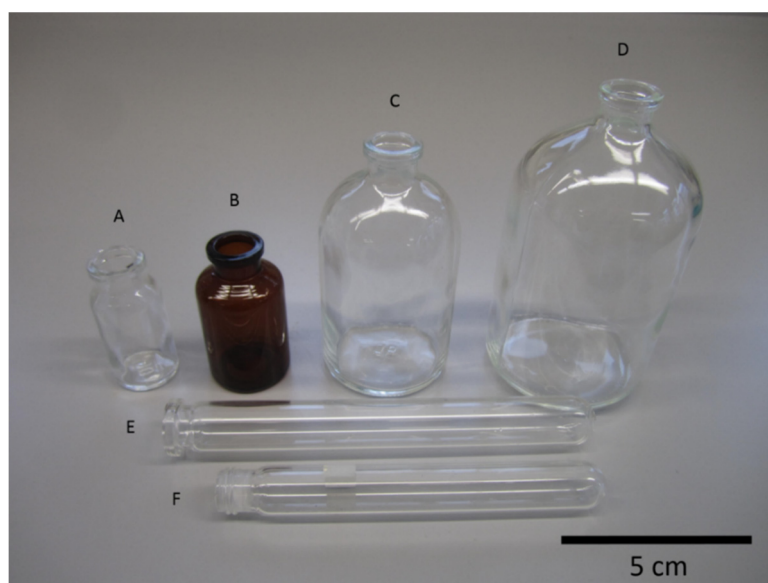
Figure 7 Vials for cultivation and medium: Bottle A (10 mL), bottle B (20 mL, brown), bottle C (100 mL), bottle D (200 mL), tube E (Balch-type), and tube F (Bellco-type).

Six different bottles were used for cultivation and medium (figure 7). All bottles are guaranteed against breakage due to pressure (2.5 bar). Bottle A (10 mL) was used for the roll-tube technique for cultivation on the solid medium. Brown bottle B (20 mL) was used for the storage of antibiotics. Bottle C (100 mL) was used for the cultivation of monoculture (50 mL working volume to ensure sufficient gas phase) to produce a relatively large amount of cell mass and DNA for genome sequencing. However, high cell density could be obtained in tube E including 5–10 mL medium. Bottle C could also be used for medium components like acetate, trace elements, and vitamin solution in anaerobic and sterile conditions. Bottle D (200 mL) was used for cultivation medium storage. Tube E was generally used for cultivation in liquid or cultivation with an angular agar medium. Bottles A-E can be sealed with blue butyl rubber stopper (gas tight, figure 8, B) and an aluminium cap. Tube F (Bellco) was seldom used because the black butyl rubber stopper was no longer gas tight after several uses.
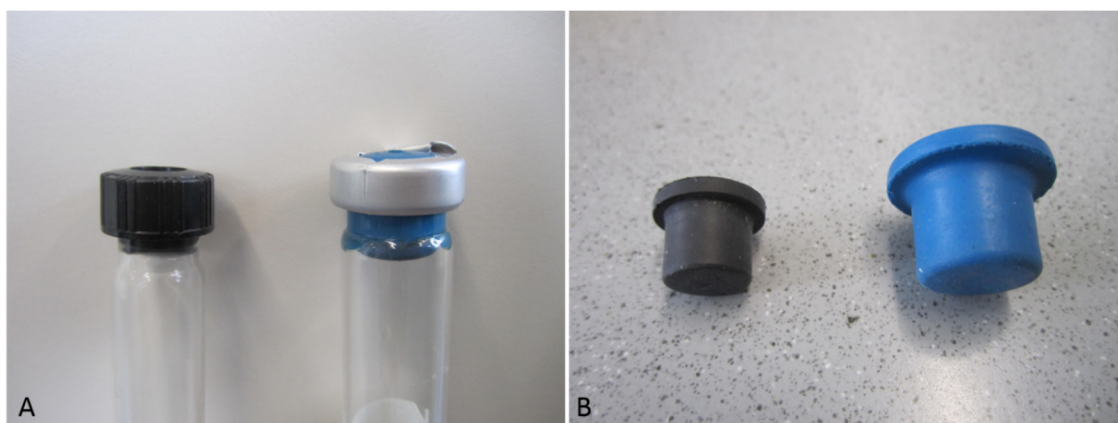
Figure 8 Two types of sealing. Left: Common black butyl rubber stopper, right: Special blue butyl rubber stopper

There are two types of sealing: Bellco-type (left) with a black butyl rubber stopper (screwable, Hungate et al. 1966) and Balch-type (right) with a blue butyl rubber stopper (Dunn, Asbach, Germany) and an aluminium cap. The blue rubber stopper was essentially more stable than the black one. Neoprene can also be used, but other rubber material like natural rubber was not gas-tight after puncturing several times with a needle.

2.2.3. Cultivation of methanogens

Cultivation was carried out in Hungate-type tube (Bellco Glass Inc. Vineland, USA) or Balch-type tube. Here, 4.5 mL medium was used and 0.5 mL inoculum was added. Gas mix (product name: hydrogen N30 and carbon dioxide N45, 80%/20%, company Air

liquide, Stelle, Germany) was fed up to a level of 2 bar overpressure, to cultivate hydrogenotrophic methanogens. Hydrogen gas could be produced by equipment using electrolysis (PACKARD, SL-9200-8, Middelburg, Germany) in our laboratory, but it did not seem to be suitable for cultivating strict anaerobic methanogens in terms of the gas quality i.e. the toxicity of oxygen. Cultivation was performed at four different temperatures: 20°C, 26°C, 37°C and 53°C, in order to separate temperature-specific methanogens. Generally, the growth rate is lower at low temperatures than at higher temperatures, but this also depends on species and activity (exponential growth or stagnation state). In general, at the higher temperature of 53°C, morphological diversity was lower, e.g. exclusively methanogenic rods were normally found. During the cultivation, gas pressure, turbidity of cells were checked and the culture was microscopically verified for purity. When measuring the gas pressure or at feeding or sampling, air can be introduced into the tube. As a result, the medium colour can change (oxidise) to pink because of the redox indicator resazurin. In such cases, 50-100 µL of hydrogen sulphide or titan citrate solution was added.

Reference species from DSMZ

For the optimisation of the cultivation and isolation system, some reference species from DMSZ (German collection of microorganisms and cell cultures, www.dsmz.de) were used. (DSM1498 *Methanoculleus marisnigri*, DSM3915 *Methanoculleus thermophilus*, DSM3267 *Methanothermobacter thermoautotrophicus*, DSM 2133 *Methanothermobacter marburgensis*). By cultivation of these species, the optimal condition of each species could be tested in various medium components.

Inoculation, transfer, dilution series

Inoculum was taken from 14 different anaerobic biogas digesters which were fed with different substrates (table 2). Normally, 10% was inoculated aseptically in the medium using a syringe. Afterwards, oxygen was excluded and cysteine and sodium sulphide (or titan citrate) solution was added.

Table 2 Inoculum for enrichment series

Number	Source (biogas plant name)	Substrate of biogas plant, Temperature
1	Hocke	Cattle manure, maize, 37°C
2	Viersen	Pig manure, maize, 53°C
3	Biowerk	Food waste, 37°C
4	Kümpers	Cattle manure, maize, 37°C
5	Ernesto	Maize, triticale, 37°C
6	Bützberg	Food waste, 37°C
7	Dario	Maize, triticale, 37°C
8	Geesthacht	Sewage sludge, 37°C
9	Danielle	Maize, triticale, 37°C
10	Antonio	Maize, triticale, 37°C
11	Seth	Cattle manure, maize, 53°C
12	Luca	Maize, triticale, 37°C
13	RM60 (HAW)	Fodder beet, 60°C
14	Fuzzy1 (HAW)	Straw, 37°C

Transfer and dilution series for the enrichment of target methanogens were performed according to the Hungate technique, under anaerobic N_2 flushing. After every transfer and dilution series, oxygen was excluded and reducing agents were added. H_2-CO_2 was added up to 2 bar overpressure. Thereby the remaining amount of N_2 was left in cultivating vessels. Dilution series were conducted ten times, normally up to 10^{-3} or 10^{-5} depending on cell density. While N_2-flushing simultaneously in two sterile tubes, the dilution series was performed in anaerobic conditions (figure 9). After dilution, the tubes were closed quickly. Oxygen was then rapidly excluded. Cysteine solution (100 µL) was added. Finally, H_2-CO_2 was fed in.
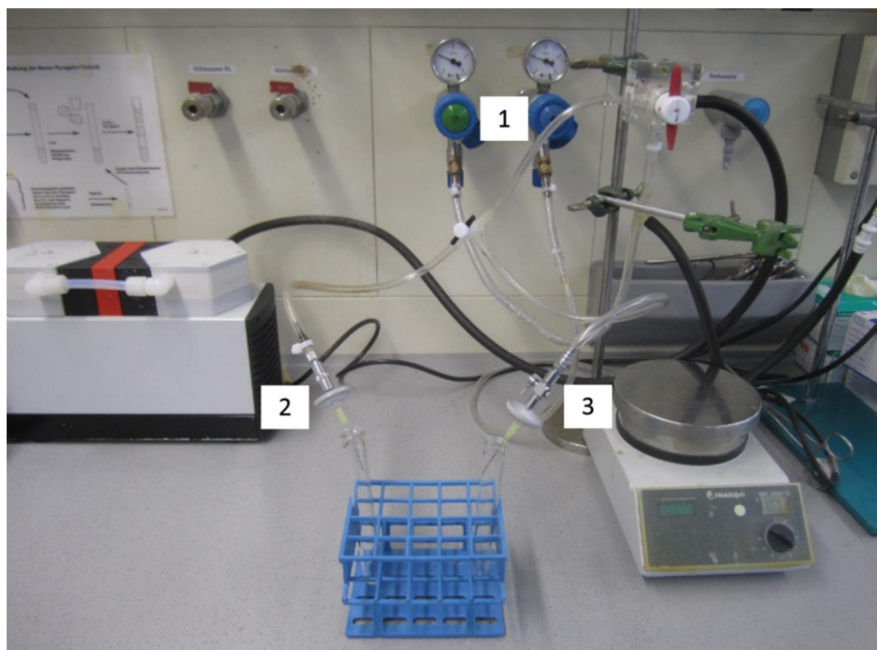
Figure 9 Equipment for dilution series under N₂ flushing. 1: N₂ source. 2 and 3: Two hoses with sterile filter and cannula for flushing two culture tubes

2.2.4. Isolation of methanogens

Antibiotics were applied, such as vancomycin (100 µg/mL, Kamagata and Mikami 1991), cycloserin (Ferguson and Mah 1983), cefoxitin (200 µg/mL, Harris at al. 1984), cefsulodin, cefotaxime, chloramphenicol, ampicillin, rifampicin, and bacitracin (15 µg/mL, Stantscheff et al. 2014) or combinations of two or five of the above (see appendix) for the inhibition of bacteria. Cell wall synthesis or protein synthesis of non-target bacteria should be inhibited by the antibiotics. Generally, antibiotics worked well but some bacteria (short rod-type, highly motile, always odoriferous when these bacteria were found) were very resistant.
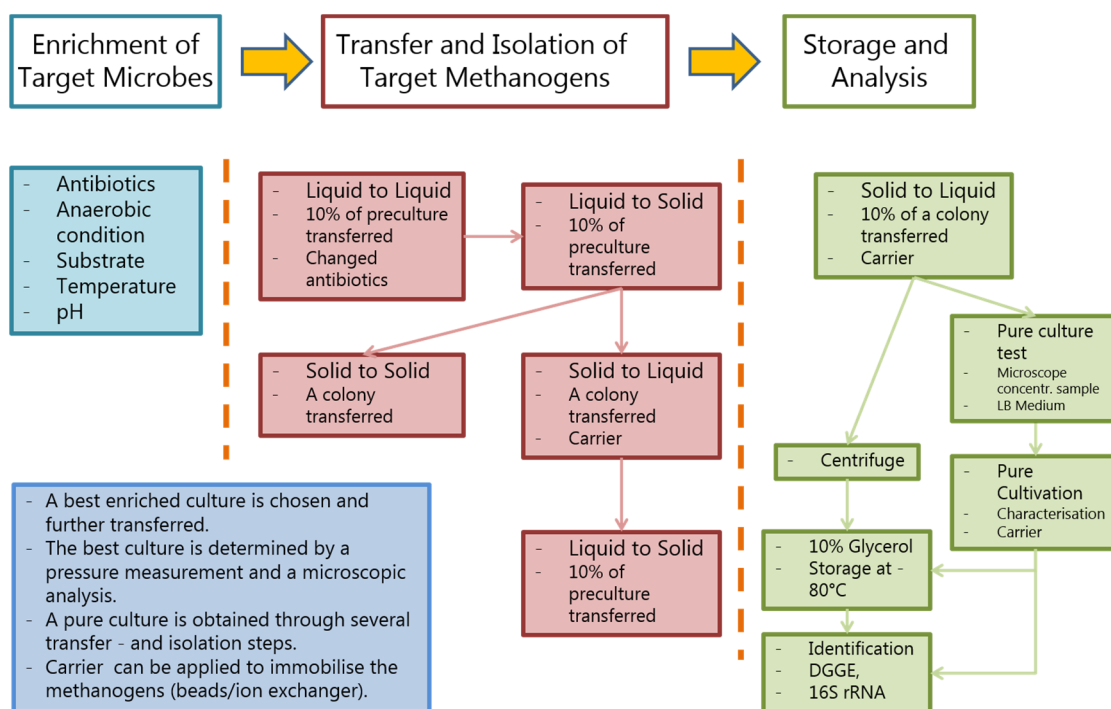
Figure 10 Summary of possible isolation strategies to obtain new methanogens. The method 'liquid to liquid' was used in general.

For the enrichment of target methanogens as well as the exclusion of bacteria, the culture was transferred (7–10 times) and diluted max. 1000 times from liquid to liquid culture, which was mainly used (figure 10, see also section 4.1.). At each step, growth took ca. 3-6 days for liquid culture or 15 – 30 days for solid media. After the removal of bacteria, separation of target methanogens from other methanogens had to be performed. This task was sometimes more difficult. In particular, the separation of coccoid-type methanogens from rod-type methanogens was revealed to be very difficult. After lengthy cultivation, the morphological mono-culture of coccoid-type methanogens was contaminated by rod-type methanogens. However, several strategies were performed (table 3) for this purpose. The purity of isolates was tested by several methods such as addition of yeast extract, amplification with bacterial and archaeal primers and sequencing by 16S rRNA.

Table 3 Strategies for the separation of target methanogens from other methanogens

Temperature change	pH change	Nutrients change
The culture was moved from low temperature to high temperature or vice versa	The culture was moved from pH 7.0 to pH 6.4 or vice versa	NaCl, KCl, NH_4Cl, $MgCl_2$, $CaCl_2$, and trace elements or their combination were added.
The methanogens are very sensitive to temperature. Methanogens that are in favour of the used temperature, will be dominant. This method seemed to be the most efficient way to isolate target methanogens	For the separation of coccoid-type methanogens from rod-type methanogens.	For the separation of coccoid-type methanogens (*Methanoculleus*) from rod-type methanogens.

Isolation of methanogens from liquid medium

For the isolation of target methanogens in liquid medium, dilution series and repetition of the transfer to new medium were conducted. During this procedure, the most dominant methanogens were left. At every transfer or dilution step, 3-5 antibiotics were added. Depending on the species, each cultivation took 3-7 days and growth of the culture was confirmed microscopically. After the 5-7th transfer, monoculture was obtained. Various temperature, pH, media and inoculum conditions were tested for this purpose. It was a relatively simple method compared to the isolation of methanogens from solid medium. A disadvantage of this method was that similar methanogens were often isolated, which often occurred as fast growing and dominating methanogens. To obtain new methanogenic isolates, a new method, e.g. isolation from a solid medium or a new medium components, should be tested.

Isolation of methanogens from solid medium

Isolation of target methanogens from solid media was generally much more difficult because the methanogens did not grow well on the solid medium in this study. Several hundred attempts failed in our laboratory. Anaerobic bacteria grew but methanogens did not (or very seldom). However, during this PhD work, for two years, the isolation method from solid medium was developed and further optimised.
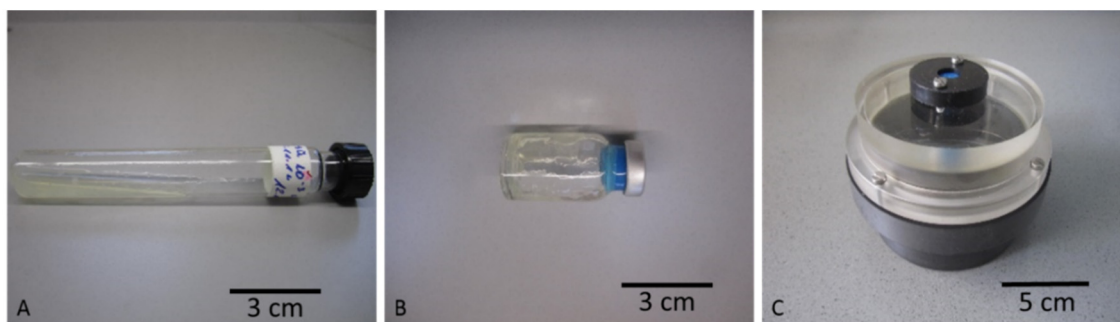
Figure 11 Three types of cultivation vessels for solid medium

For cultivation on solid medium and easy access to picking up the colony, three different techniques were applied (figure 11 A: angular agar medium, B: roll-tube technique (10 mL serum flask), C: anaerobic petri-dish box). All of the culture systems were appropriate for the cultivation of hydrogenotrophs in overpressure of hydrogen up to 2 bar. Thereby, a remaining amount of N_2 was left in the culture vessels. Depending on the species, each cultivation took 10-30 days and growth of the culture was confirmed microscopically.

The solid medium was prepared differently to table 1, as detailed below (table 4).

Table 4 Preparation of solid medium used for all growth temperatures 26°C – 53°C

Angular agar medium (100 mL)	Roll-tube technique (20 mL)	Anaerobic petri-dish box (20 mL)
A 93 mL macro element solution was prepared in a 200 mL beaker. 1 mL imidazole solution, 1 mL acetate solution, 1 mL formate solution were added. Agar (1.8%, 1.8g), yeast extract (0.5%, 0.5g), tryptone (0.5%, 0.5g) and 200 µL resazurin were added as well. Oxygen was excluded, it was autoclaved and stored in 60°C water bath. Then, 4.8 mL of the above solution was anaerobically transferred in to 10 mL anaerobic sterile tubes (totally 12 tubes) at 60°C. Next, 200 µL cysteine, 400 µL sodium sulphide, 1 mL vitamin, 1 mL trace elements, 500 µL polyphosphate, and 100 µL titan citrate solution were placed in another 10 mL anaerobic sterile tube. From this solution in the 10 mL tube, 200 µL of aliquot was added to the 4.8 mL medium-tube prepared before. H_2-CO_2 was added up to 2 bar overpressure. The tubes were placed sloped while agar became hard.	10 mL HAW medium was added to a 25 mL Schott flask. Agar (1.5%, 0.15g), gelrite (0.5%, 0.05g), yeast extract (0.5%, 0.05g), trypticate (0.5%, 0.05g) were added to the medium. This solution was autoclaved and stored in a 60°C water bath. 10% of a culture, which had been stored in 60°C, was inoculated and mixed in water bath. About 3 mL of the inoculated medium was poured in a 10 mL sterile serum flask and closed with a butyl rubber stopper. The flask was rotated (10-15 seconds) by hand while the medium was fixed on the inner wall of the flask. Inside space of the flask was empty which enabled picking colonies easier. Oxygen was excluded and reducing agent (100µL Na_2S) was given. H_2-CO_2 was filled up to 2 bar overpressure.	20 mL HAW medium was added to a 25 mL Schott flask. Agar (1.5%, 0.15g), trypticate (0.5%, 0.05g), yeast extract (0.5%, 0.05g) were added to the medium. The solution was autoclaved and stored in a 60°C water bath. In a sterile bank, 200 µL inoculum, 50 µL sodium sulphide, 50 µL cysteine, 50 µL titan citrate, 200 µL vitamin, 200 µL trace elements were quickly added. The anaerobic box was closed and oxygen was excluded. Reducing agent (100µL Na_2S) was added. H_2-CO_2 was filled up to 2 bar overpressure. If the medium became pink, reducing agents were added again.

Inoculum could be added directly to the medium in a water bath or added to the medium anaerobically after agar became hard. Although water condensate was removed through the rubber stopper, colonies were often growing together (not as a single colony) on the angular agar medium (A). Reference methanogens DSM3267 *Methanothermobacter*

thermoautotrophicus could be cultivated on the medium used for the roll-tube technique (B).

During the study, a gas-pressurised anaerobic petri-dish box was developed (figure 12). This is a box for a single petri-dish. Regular gas feeding up to 2 bar overpressure is possible. It was gas-tight for up to 3 months. The material used was polyoxymethylene (POM) for the lower part and polymethyl methacrylate (PMMA) for the upper part; the box was thermostable up to 100°C. The upper part was transparent, which enabled colonies to be detected without opening the box. The upper and lower parts were sterilised by UV-light and disinfectants, respectively.
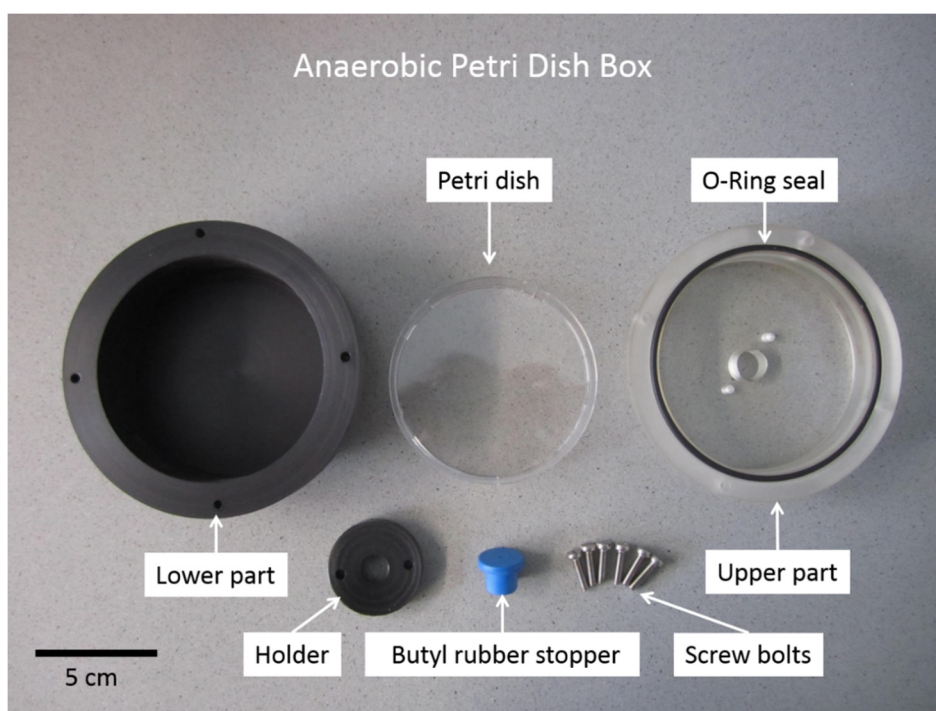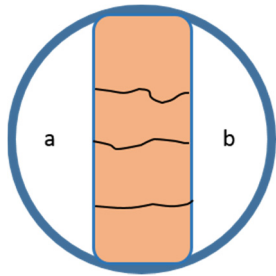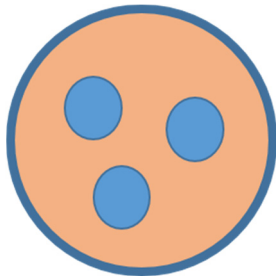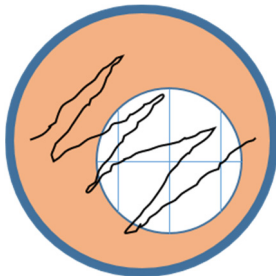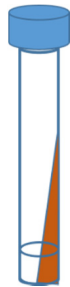
Figure 12 Anaerobic petri-dish box for isolation of hydrogenotrophic methanogens developed during PhD work

With the anaerobic petri-dish box and angular agar medium tube, various cultivation variables could also be tested (table 5).

Table 5 Four variations of the cultivation on the solid medium

Anaerobic petri-dish box (top view)			Angular agar medium tube (side view)
Thin drains on a agar barrier between two liquid media a/b in a petri-dish	Holes in the agar in a petri-dish	Filter paper on the agar in a petri-dish	Angular agar medium in artificial swamp
A mix culture was put on side 'a' in the liquid medium. On the opposite side 'b', e.g. there is a liquid medium with more nutrients attracting target movable methanogens from side 'a' to 'b', e.g. a liquid medium with acetate was put in side 'b'. Agar medium blocked between side 'a' and side 'b'. Three routes (drains made by a cannula with width approx. 10 µm) on the agar were made for the target methanogens to move from side 'a' to side 'b'. In side 'b', the target methanogens should be enriched.	Three holes were made in the agar medium and filled with liquid medium in order to supply the easily available liquid nutrients lastingly.	100 µL of a culture was spread out. A filter paper (pore size 0.25 µm) was put on the spread culture on the agar, through which only liquid nutrients could penetrate. Afterwards, liquid medium was poured on the filter paper in order to supply the easily available liquid nutrients.	Angular agar medium was made. Additionally medium with sodium sulphide was given to make a swamp conditions artificially. The tube was saved vertically.

In terms of oxygen toxicity, preparation of the agar medium and its transfer to the anaerobic petri-dish box was improved (figure 13).

More improved preparation of agar medium for the anaerobic petri-dish box
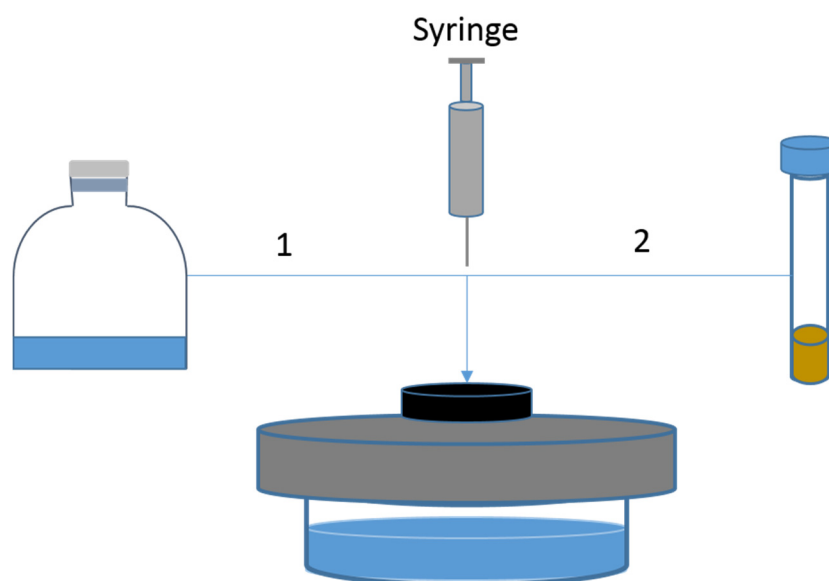
Figure 13 Solid medium was prepared and inoculation was conducted without any contact with oxygen

As indicated in figure 13, 20 mL agar medium (1) was prepared anaerobically and autoclaved in a 100 mL serum flask with a butyl rubber stopper. Oxygen was excluded (see 2.2.3). The agar medium was saved in 60°C water bath after autoclaving. After being autoclaved, oxygen was again excluded from the liquid agar medium. Foam formation could occur during boiling of the medium; for this reason, the 100 mL serum flask was used. Here, 150 μL of titan citrate was added to the agar medium. On the other hand, heat-sensitive medium and possible chemicals causing precipitation, like vitamins (200 μL), trace elements (200 μL), cysteine (50 μL), Na_2S (50 μL), titan citrate (100 μL) and inclusive inoculum (500 μL), were anaerobically prepared in another tube (2) at room temperature in figure 13. Oxygen had been previously removed from the anaerobic petri dish box and stored with H_2S (1 mM) for one day by a syringe which had also been flushed by N_2. Afterwards, the liquid agar medium was pipetted into the anaerobic petri-dish box by a syringe (2.1x80 mm, 3*1/8″). Oxygen had been excluded again from the anaerobic petri-dish box. The anaerobic box was placed on the thermo-plate at 60°C and stirred magnetically. The thermo-plate was turned off and the inoculated agar medium became fixed. The temperature decreased to the desired cultivation temperature 53°C or 37°C. The medium mixture with inoculum was put into the anaerobic petri dish box by a syringe. The most important advantage of this system was that all procedures were performed anaerobically without opening any cover.

Risk of contamination by bacteria

Contamination by bacteria during the work must be avoided. There were some positions in which contamination might happen: the cannula for gas feeding, the cannula for the exclusion of oxygen, the cannula for reducing agents, and the cannula for medium, as the same cannula was used for different cultures. Therefore, the cannula should be often replaced and the surface of the rubber stoppers should be disinfected by 70% ethanol. In this study, two types of bacteria (spiral and short rod-type) caused repeated contamination. Both bacteria were very actively motile and relatively resistant to antibiotics.

Risk of contamination by other methanogens

Bacteria could be removed by antibiotics. However, contamination with non-target methanogens would be problematic. It was often observed that coccoid-type methanogens were dominant, like the mono-culture proved by 16s rRNA in the beginning, but some rod-type methanogens occurred occasionally during cultivation because the rod-type methanogens seemed to be more robust to starvation condition (Cho et al. 2013).

2.2.5. Technical improvements for anaerobic cultivation

A methodical development and modification was performed. Synthetic medium was used and optimised in order to enrich methanogens selectively, which was a fundamental factor for successful isolation. For the removal of oxygen from media, a gassing/degassing system using a vacuum pump and inert pure gas N_2 was used, without a copper oxide as oxygen scrubber (Hungate 1969). Various media and antibiotic tests were carried out using anaerobic reaction tubes. To obtain isolates, three different types of solid medium vessels were used (figure 11); thereby, the second one was a modified Hungate roll-tube technique (Hungate 1969) and the third one was the self-developed anaerobic petri-dish box. This anaerobic petri-dish box was assembled by the manufacture of HAW; it was gas-tight. Regular gas feeding was possible. Picking colonies became much easier. The detection of colonies was possible, as shown in figure 17. Gas feeding of the culture tubes with 2 bar (200 kPa) overpressure, and the aseptic addition of medium additives is demonstrated in figure 14. Picking of a colony in a serum flask is shown in figure 16.
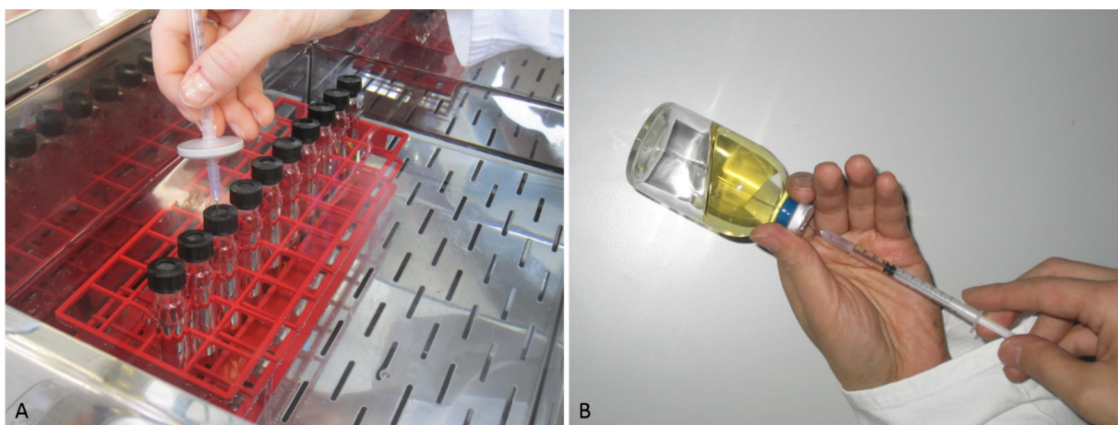
Figure 14 Gas feeding of culture tubes (A) and aseptically adding the medium to a 100 mL bottle (B)

All gas feeding tubes were connected with a filter (0.2 µm pore size, PTFE hydrophobic) at the end of the tube. This filter was further connected with a commercially available, sterile cannula. All media were transferred using sterile syringes (1 mL, 3 mL, 5 mL or 10 mL) with sterile cannulas (0.9 x 40 mm and 0.55 x 25 mm). All steps were carried out anaerobically without an anaerobic chamber or anaerobic jar, which can incur a significant cost and is a possible explosion risk.
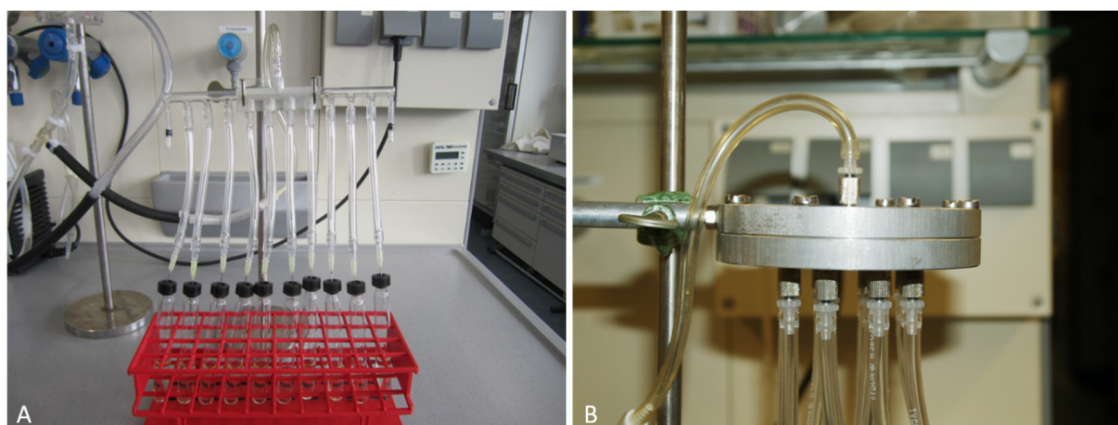
Figure 15 Previous manifold (A) and improved new manifold system (B) for exclusion of oxygen from culture tubes

For the exclusion of oxygen from many tubes simultaneously, a gas manifold was needed (figure 15). With the previous system (A), it was difficult to induce overpressure or the

vacuum state due to weak connections. Thus, a new gas manifold with a metallic Luer lock system was assembled (B). This was gas-tight to overpressures of up to 1.5 bar.
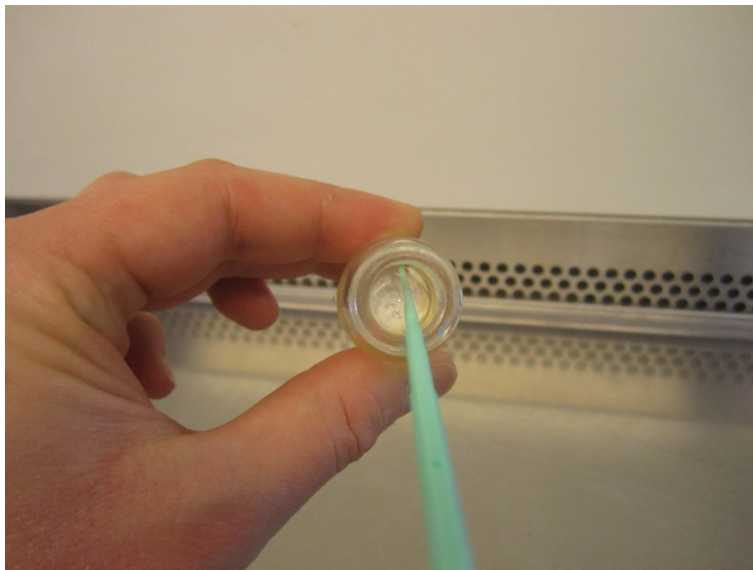
Figure 16 Picking a colony in a roll-tube (10 mL serum bottle)

Picking the colony was carried out using a sterile plastic loop or a sterile cannula in a sterile bank or under N_2 flushing in anaerobic conditions. One problem was that the chosen colony was normally not as large as necessary to transfer it into liquid medium and microscopic analysis simultaneously. A single colony was normally just a few millimetres in diameter. The colony was therefore not big enough to be divided into two for the above purposes. It was also not always easy to dissolve it in a liquid medium. Mixing by vortex was often insufficient to suspend the colony in the liquid medium. Thus, a strategy was developed. Firstly, the colony was picked and put on a sterile microscope slide. The colony was suspended in 10 μL sterile water on the slide by a sterile plastic loop. The plastic loop used for suspending the colony was used again for inoculating in a sterile liquid medium. The remnant culture on the microscope slide was analysed under a microscope. Consequently, both goals were achieved for even small colonies.
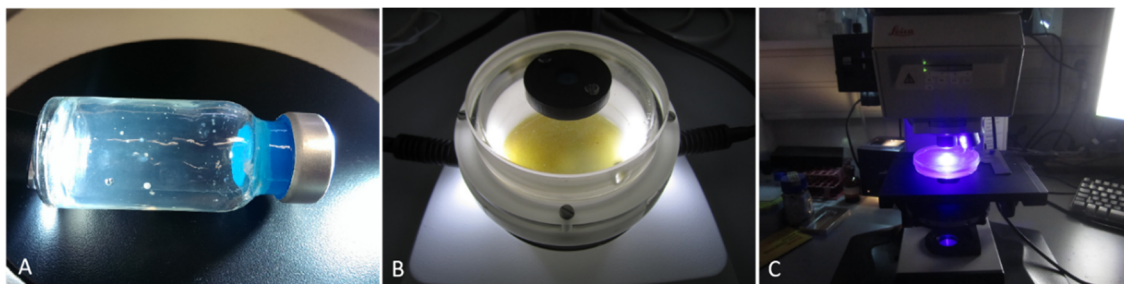
Figure 17 Visualization of a grown colony in a roll-tube (A), in a petri dish box (B) and a petri dish (C)

Colonies were formed and detected in a 10 mL serum bottle (A) and an anaerobic petri-dish box (B) in figure 17. However, no methanogenic colony could be differentiated from other bacterial colonies. A UV-pocket lamp (wavelength 400 nm, 3W) was tested to detect the colonies (Doddema and Vogels 1978); however, it failed because the wall of the bottle and the plastic cover was so thick and the UV light could not clearly penetrate. Thus, a detection method (C) was designed. The petri-dish in an anaerobic petri-dish box, where colonies occurred, was taken out and promptly closed with a sterile petri-dish cover. This covered petri-dish was again sealed at the side with a parafilm. The sealed petri-dish was then brought to the table of a fluorescence microscope. The colony was detected under 40x magnification; first by light microscopy and second by fluorescence microscopy (wavelength 420 nm). The colony which shines in the mode of phase contrast as well as fluorescence mode would be the methanogenic colony which could be selected and confirmed under 400x magnification at level of a single cell.

The developed and improved methodology can be used for universal application of cultivation and isolation of strict anaerobic microorganisms. The system is simple and cheap, gas-tight and proper for regular gas feeding. Colonies are easily detected. Access to a colony is easy. After picking, the anaerobic box can be further used or the used petri-dish can be changed. The system is also pressure- and thermostable.
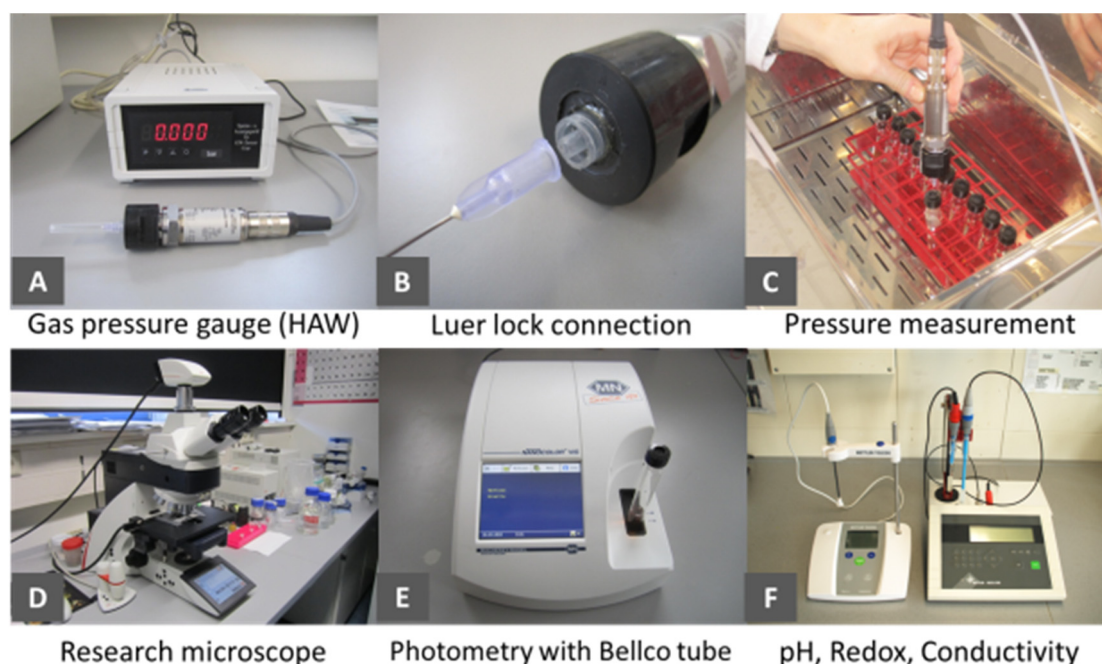
Figure 18 Equipment to measure gas pressure (A, B, C), presence of methanogens and their growth (D, E). Desk-instruments to measure the pH, redox potential and conductivity of culture media (F)

Growth of hydrogenotrophic H_2-CO_2-consuming methanogens could be monitored by measuring pressure. When bio-methane is produced, the total pressure of the gas phase in cultivation vials is decreased according to the following biochemical reaction: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$. In this way, methanogenic activity was measured by a gas pressure gauge (A) (Special Instruments, Product number 1908, Nördlingen, Germany), which was exclusively manufactured for the lab to measure the pressure of anaerobic tubes or serum bottles. The head of manometer (B) was configured with a Luer-lock which can easily be connected to a commercial Luer-lock cannula. Gas pressure measurements (C) were done in a 60°C water bath due to the changeable gas volume depending on temperature. Fluorescence microscopy (D, Leica 6000B) was used to confirm the presence and quantity of methanogens (Doddema 1978) as well as their morphotypes, and the purity of culture. Cell concentration could be also measured with a spectrometer for optical density (E, Nanocolour VIS, Macherey-Nagel, Düren, Germany). But as other particles interfered, it was not often used. Furthermore, pH value, redox potential and conductivity (4 mm electrode diameter for microreaction tubes, Eppendorf, Hamburg) was checked (F) for optimal medium.

DNA extraction and PCR amplification from the isolates

Total DNA was extracted from 1-2 ml of liquid enrichment culture using the Gene Matrix Stool DNA Purification Kit (Roboklon, Berlin, Germany) according to the manufacturer's instructions. PCR amplification of 16S rRNA genes was performed in 50 µl reactions containing: in total 3.5 mM $MgCl_2$, 1.0 µM of each primer, 0.24 mM of each dNTP and 1.7 Unit Dream Taq Green DNA polymerase (Fermentas GmbH Part of Thermo Scientific, St. Leon-Rot, Germany) with 1 x buffer and 3 µl template DNA. Two PCR primer sets, 27F-1492R for bacteria (Lane 1991) and A337F-A1000R for Archaea (A337F, 5´-TAYGGGGYGCAGCAG-3´, modified Gantner et al. 2011; A1000R, 5´-GAGAGGAGGTGCATGGCC-3´, Gantner et al. 2011; seller: Eurofins Genomics, Ebersberg, Germany) were used. The PCR program consisted of a single cycle of 96 °C for 3 min, followed by 30 cycles of 96 °C for 50 s, 53 °C for 50 s, and 72 °C for 90 s. A final extension was performed at 72 °C for 8 min. PCR amplicons were purified with the Gel extraction/PCR clean-up Kit (Geneaid Biotech Ltd., Taiwan; seller: DNA Cloning Service, Hamburg, Germany). The archaeal 16S rRNA gene fragments were sequenced by the company GATC (Köln, Germany) or LGC (Berlin, Germany) using the Arch915R primer (5'-TGCTCCCCCGCCAATTCCT-3', Tokura et al. 1999; seller: Eurofins Genomics, Ebersberg, Germany). The obtained 16S rRNA gene sequences were compared with those on publicly accessible databases by using the program Basic Local Alignment Search Tool (BLAST; NCBI) (Altschul et al. 1990). This molecular analysis was performed by Dr. Sandra Off (Hamburg University of Applied Sciences).

Chapter 3: Results and Discussion for Fluorescence Microscopy and Image Analysis

The true sign of intelligence is not knowledge but imagination

Albert Einstein

3. Results and discussion of the developed microscopy

3.1. Validation of the cell counting technique

Fluorescence microscopy was used for the detection of methanogens as well as the determination of their autofluorescence intensity based on coenzyme F_{420}. First of all, dependency of the general fluorescence intensity was investigated. In a study it was shown that integrated fluorescence intensity could be dependent and proportional to e.g. the number of hybridised probes (Poon et al. 1999). A cytometry study showed that a relative mean fluorescence intensity can be used as a prognostic marker in an immunodeficiency disease (Liu et al. 1996). In this study, auto-fluorescence intensity of methanogens was used for an indicator for methanogenic activity. For this purpose, basic experiments regarding the optimal fluorescence light source setting and segmentation of objects from fluorescence background were performed.

Segmentation of objects from fluorescence background

Fluorescence intensity can depend on the strength of the light source, signal intensification (amplification), filter cube, and exposure time (Masters et al. 1997; Poon et al. 1999) as well as on the quality in focusing and timing of image acquisition.

Equation	y = a + b*x		
Adj. R-Square	0.93549		
		Value	Standard Error
Fluorescence intensity	Intercept	0.6	8.22679
Fluorescence intensity	Slope	25.8	3.35857
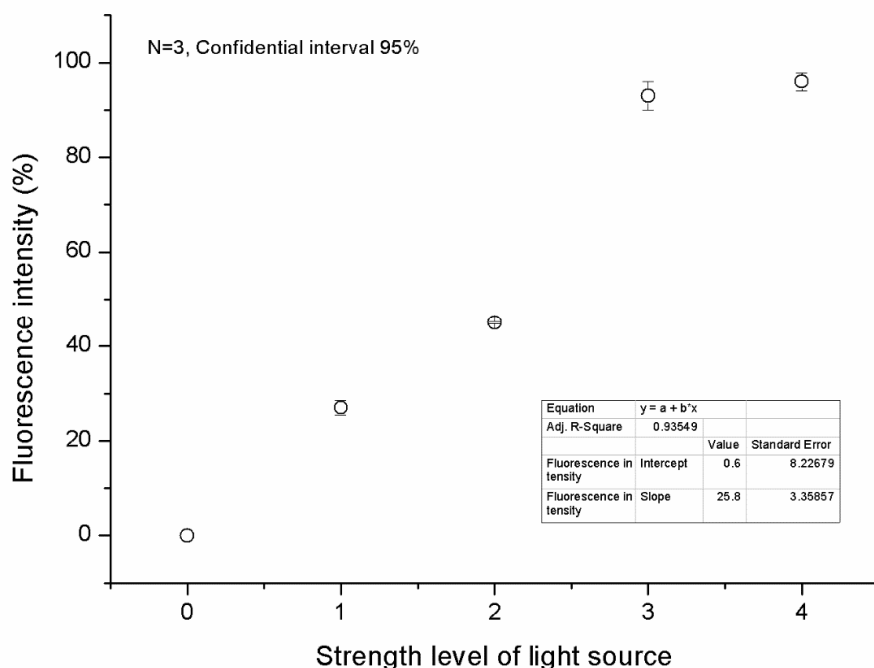
Figure 19 Fluorescence intensity of a standard fluo-bead in relation to different strength levels of the microscope light source

First of all, dependency of the strength level of the light source on the fluorescence intensity was tested (figure 19). The intensity of a green fluorescent bead (fluo-spheres, 4 μm, F8859, Life Technologies, Darmstadt) was measured in triplicate as a reference at four different strengths of light source (Leica EL6000, using an alignment-free, mercury metal halide bulb). Biological fluorescent material could not be used because they rapidly lost the intensity. In this experiment, optimisation of the level of light source strength was focused. By using e.g. DAPCO-solution as anti-fading agent, bleaching out effect of biological samples could be significantly reduced. There was a linearly proportional relationship (r^2 = 0.94) between strength level of the light source and fluorescence intensity. A slight deviation was also shown for the three repetitions. As we aimed to measure 100% intensity as a set point, the strength of the light source was fixed at level 4 for all measurements. The intensity could also be dependent on the lifetime of the light source, but this was not tested due to time limitations. Ideally, absolute fluorescence intensity of target objects should be measured. However, this depends on many factors like objects themselves, the matrix of the medium, the microscope, the camera and the light source (Poon et al. 1999), which means that it is impractical for daily analysis. Absolute fluorescence intensity could be measured by a single photon counting photomultiplier tube (Masters et al. 1997). There is also a microscopic calibration slide for the measurement of absolute fluorescence intensity available (www.argolight.com, France), which can be used as a reference for each measurement. Fluorescent beads could also be used as a reference, which should be mixed with the sample for each measurement at every measurement (Poon et al. 1999). Both applications above were not realised routinely during the work due to their high costs and inconvenience of use.

Definition of fluorescence intensity used in this study

Thus, it was decided in this study that relative fluorescence intensity (1-255 or 0–100%) should be measured. The relative fluorescence intensity of an object (white) is based on the background fluorescence (black) as a reference at every measurement. Thus, the background intensity is the reference value. To the best of our knowledge and in our experience, the background was generally always black. The segmentation of an object from the background could also be improved by image analysis (figure 23). The relative fluorescence intensity only indicates the difference in intensity between objects and their background, where other factors like the matrix of the medium, the microscope, the

camera and the light source became meaningless (not influential). Theoretically, the relative value is not comparable between different samples and microscopes because it is not an absolute value. However, its difference between different samples could be negligible in the absence (or nearly absence) of other interfering fluorescent particles, which consequently allow determination of the difference in intensity only between objects and background. To our extensive experiences for more than 100000 pictures on methanogens measurement of the relative fluorescence intensity was reliable and was strongly suggested as a new parameter for indication of an activity of methanogenesis.
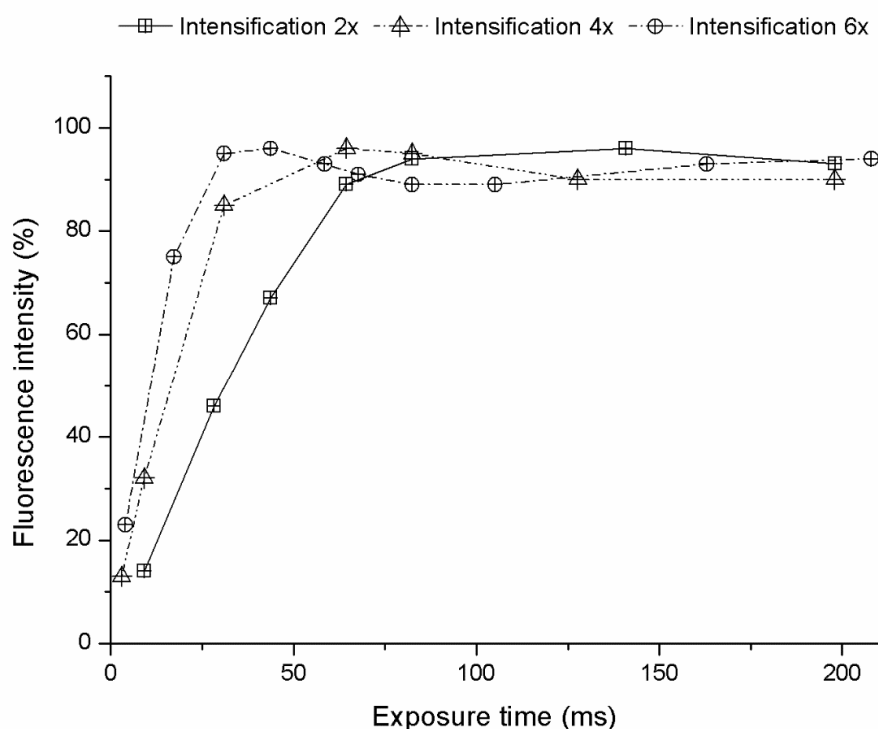
Figure 20 Optimal signal intensification factor for measurement of the fluorescence intensity of a standard fluo-bead, depending on different exposure time

Besides the strength level of the light source, the exposure time of fluorescence had to be optimised. The fluorescence intensity of a fluo-bead (fluo-spheres, 4 μm, F8859, Life Technologies, Darmstadt) was measured depending on exposure time and electronic signal intensification by the digital image analysis software Image Pro 7. The result was visualised on a monitor. During this procedure, the intensity signal can be intensified by the software. The optimal signal intensification factor for fluorescent intensity was tested (figure 20). Six times intensification reached 100% fluorescence intensity after an exposure time of about 30 ms, four times intensification at about 70 ms and two times

intensification at 70 ms exposure time. However, the slope of the two times intensification curve clearly showed a slow increase. In this study, four times signal intensification (40% of maximum) was used for all measurements.
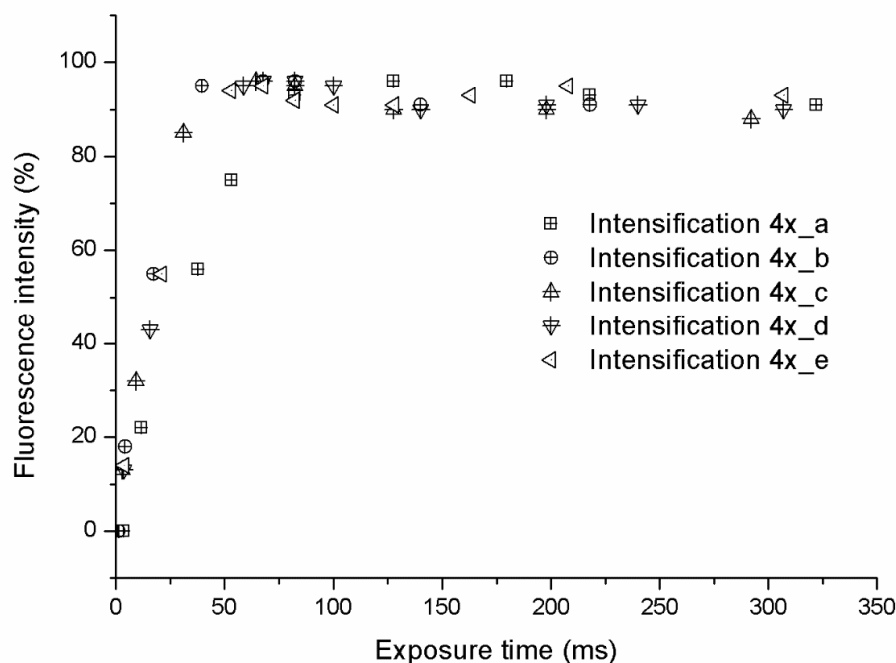
Figure 21 Optimal exposure time for measurement of fluorescence intensity of a fluo-bead

Optimal exposure time for fluorescence light of a fluo-bead (fluo-spheres, 4 μm, F8859, Life Technologies, Darmstadt) was also looked for and tested in five repetitions (a-e) at the same adjustment of the signal intensification (4x). At about 70-80 ms of exposure time, all of the fluo-beads reached almost 100% fluorescence intensity (figure 21). An exposure time of higher than 80 ms was not meaningful. Thus, an exposure time of 80 ms for the camera was fixed for all experiments in this study.

Image improvement to make a clear difference between objects and the background could be achieved using the image analysis software, in other words image filtering.
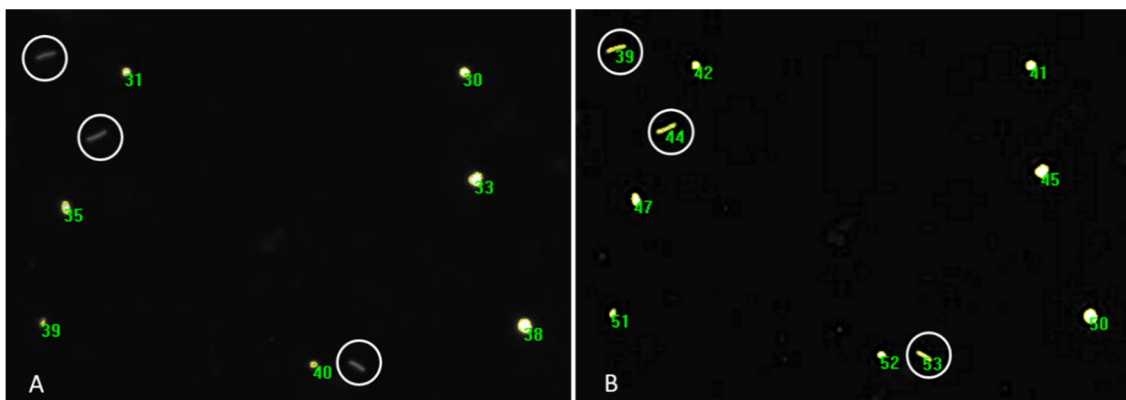
Figure 22 Automatic counting of fluorescent methanogens without image filtering (A) and with image filtering under 400x magnification

In figure 22, example images of methanogens were shown to demonstrate the effect on counts without image filtering (A) and with image filtering (B) under 400x magnification. Counting was automatically performed. In total, 57 cells in the image were counted with image filtering (A) while 53 cells were counted without image filtering (B).
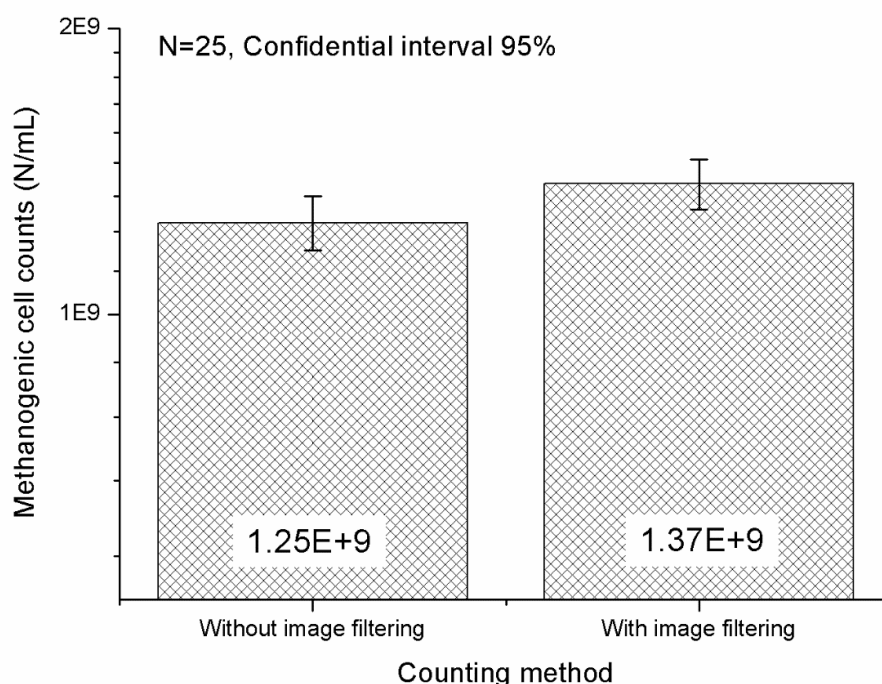
Figure 23 The result for automatic counting of figure 22, without image filtering (A) and with image filtering (B)

In figure 23, a comparison of methanogenic cell counts with and without image filtering (25 pictures) was performed. Image filtering enhanced image details with a "hi-pass filter" using a Gaussian function filter, introducing less noise. With image filtering, about 1.1-times more cells were counted than when counting without image filtering in this case.

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However, depending on the noise level of background fluorescence, the deviation will become bigger, more than 10 times (results not shown).

A sample of a biogas plant Seth could be analysed on a single slide for counting total cells and methanogens. The sample was stained with SYBR Green I. By using different wavelengths of SYBR Green and the autofluorescence of methanogens, total cells and methanogens were counted together in one step on a single microscope slide. However, accurate counting of methanogens was difficult because the cells stained with SYBR Green caused background fluorescence. In figure 24, two methods were compared: one where the sample was prepared on a single slide for the counting of total cells and methanogens, and the other where the sample was prepared on two slides separately for total cell counting and methanogenic cell counts. The first case yielded methanogenic cell counts with the fluorescence background of stained cells. The second showed cell counts that were performed separately without the fluorescence background of the stained cells.
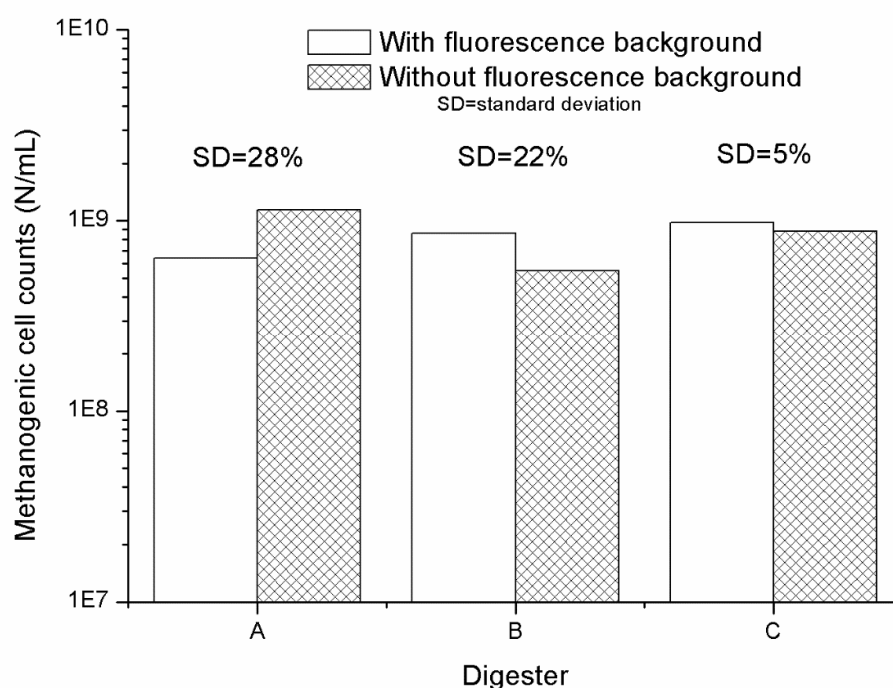
Figure 24 Comparison of methanogenic cell counts of 3 different anaerobic digesters when counting results with and without interfering fluorescence background of SYBR Green stain

Three different digester samples (A: Seth F2, B: Seth F3, C: TUAS) were measured using two different methods, i.e. one with fluorescence background and the other without fluorescence background of SYBR Green stain (figure 24). Depending on the sample, 5–

28% of deviation between two methods occurred (figure 24). This deviation could be derived from background fluorescence of the stained cells. Thus, it was concluded that methanogenic cell counts should be separately prepared and analysed on another slide without fluorescence background of the stained cells.

Storage effect on the fluorescence intensity
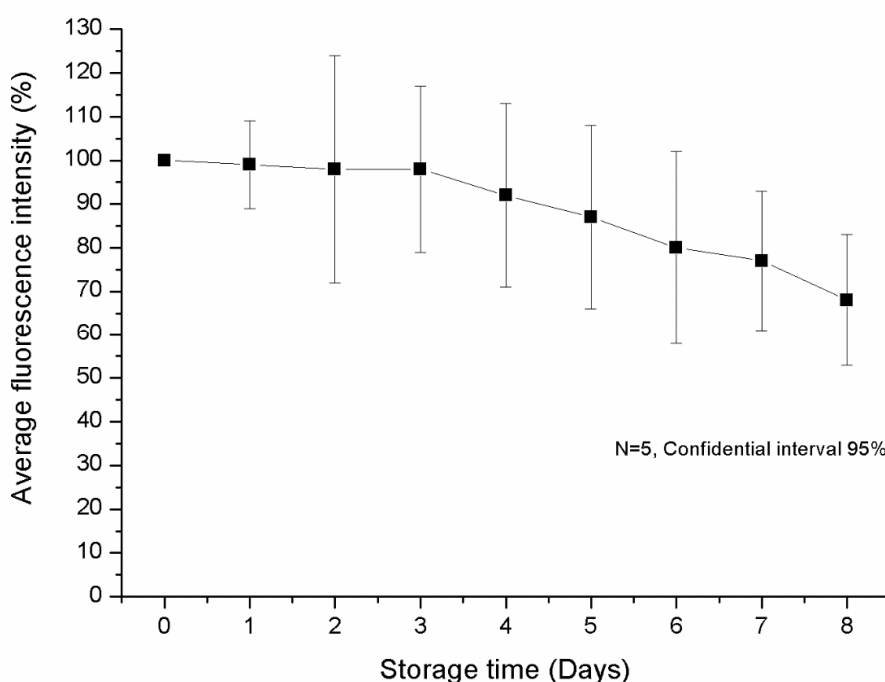
Figure 25 Decrease of fluorescence intensity over storage time

To investigate the effect of storage period on cell counts, a sample of the biogas plant Seth was prepared with methylcellulose-coated microscope slides that were covered with coverslips (see section 2.1.2.). Fluorescence intensity of the sample was measured on a daily basis. This experiment was repeated five times (N = 5). The covered slide was stored at 4°C during the daily measurements. Interestingly, up to 8 days, the methanogens were fluorescent (68%). The coverslip on the methylcellulose was supposed to protect methanogens against oxygen toxicity to some extent. Although a high deviation was shown between 5 tests (figure 25), the mean value of the fluorescence intensity was surprisingly unchanged with results of 100% for up to three days and a subsequent decrease in 5 days. Apparently, the methanogens could not grow on the sticky fixed methylcellulose. Methylcellulose is not biologically degradable. However, methanogens

were fluorescent for a while, which might mean that this activity of the fluorescent coenzyme F_{420} was related to maintenance processes. Only the oxidised form of F_{420} is fluorescent and the reduced form of F_{420} with hydrogen is not fluorescent (Thauer 1977). This result was understandable in the same context as another study where frozen methanogenic cultures were viable after 4 months (Winter 1983).

Additionally, approximately 5g aliquot of a sample of biogas plant Lanken (2L, total solid content 10.2%, stored at 4°C, maize-fed fermentor) was taken and measured on a weekly basis for 14 weeks in order to see the long-term storage effect on methanogenic cell counts. Methanogenic cell counts were 4.35E+9/mL in the first week. Interestingly the cell counts slightly fluctuated for 14 weeks around the start value of 4.35E+9/mL with standard deviation occurred ± 9.3%. The standard deviation was not depending on the time but seemed to be caused by non-homogenous sample due to a relatively high content of total solid resulted in non-perfect mixing and sampling. But the fluorescence intensity of methanogens showed a tendency to decrease over time, from 53% to 40%. In the case of a sample with lower content of total solid (below 4%), an apparent decrease of methanogenic cell counts during storage at 4°C was shown (systematic experiments were not performed).

Comparison of coating agents to fix the microbial cells in a single cell layer
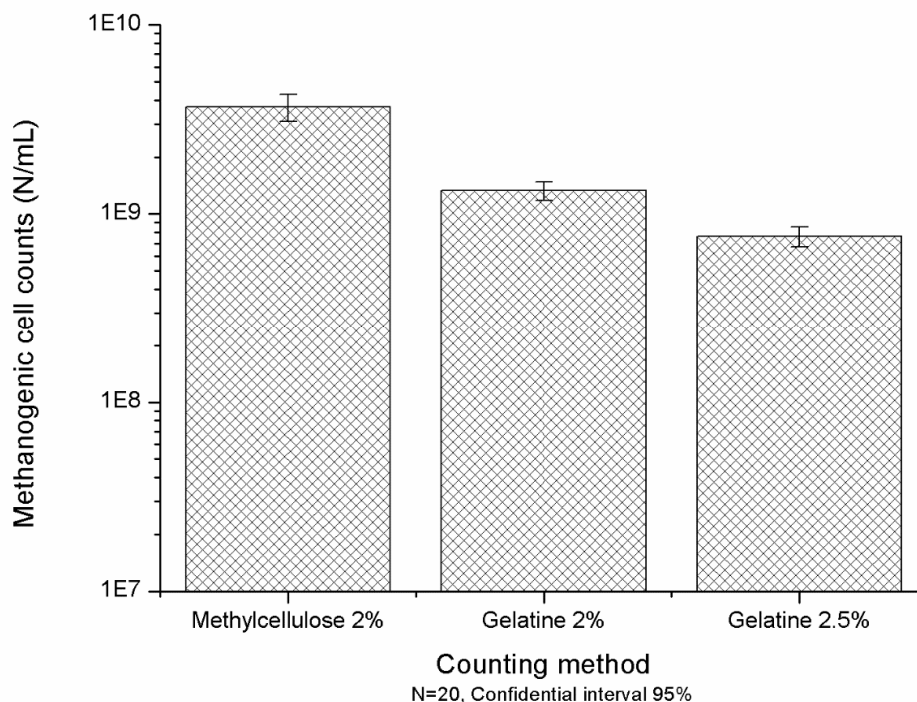
Figure 26 Comparison between gelatine and methylcellulose as a coating agent to fix microbial cells on a microscope slide

Gelatine and methylcellulose were tested as coating agents (Bast 1999). In each case, 20 pictures were taken. Generally, gelatine showed unstable characteristics and an uneven surface, resulting in non-homogenous distribution and diminished cells at counting (maximum 4-times lower than 2% methylcellulose). Agarose-coated slides could also be used (Middleton et al. 1976), but they showed much less stability and an unclear permeability of light compared to gelatine in this study (results not shown). Additionally, commercial methylcellulose is available with different viscosities. It was observed that 2% (w/w) of methylcellulose with low viscosity (400 mPas) was optimal and allowing pipetting. Methylcellulose with 1500 mPas could not be pipetted and showed less homogenous distribution. Interestingly, the high viscose methylcellulose caused cell lysis (cracking) of coccoid-type methanogens in some cases.

Standardisation of the coating procedure

Proper distribution of the coating agent was found to be essential for homogenous distribution of a sample in a single layer on a slide. It was often observed that possible deviation in this step was mostly caused by the operator using a spatula. To reduce such sources of error, equipment for distribution of the coating agent was developed during the study (figure 27).
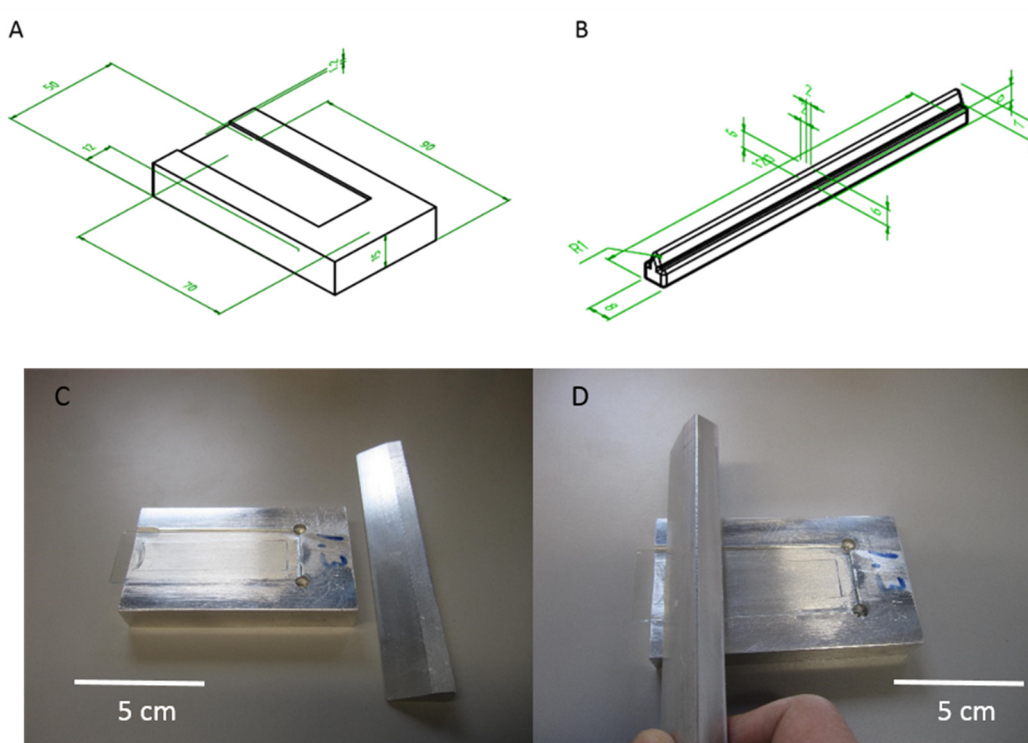
Figure 27 Construction drawing of microscopic slide holder (A, number in mm) and stick for distributing coating agent (B, number in mm), an example image of slide holder and stick (C, slender side of the stick is hold downward), and the process of homogenous distribution of the coating agent (D)

A microscope slide was put on the microscope slide holder. Then, 350 µl of methyl cellulose was pipetted onto the left corner of the microscope slide and mixed by pipetting. It was coated by moving the coating stick from the left corner to the right corner. The coating stick can be made of plastic or metal material having to be not sticky. The developed equipment improved the quality of distribution and accelerated the procedure.

Selection of image acquisition position on a sample area (blue) distributed on a microscope slide
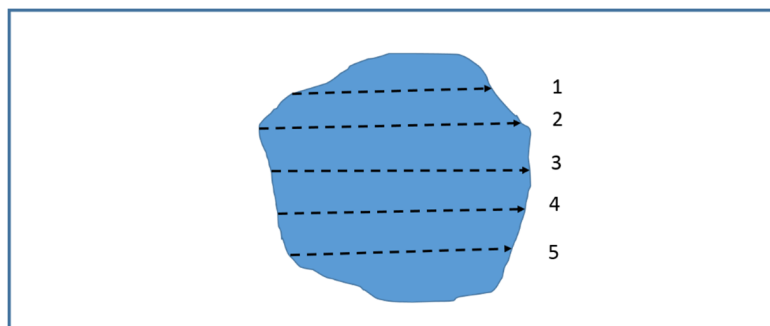
Figure 28 Imaginary model of a sample distributed on a slide: Five different image acquisition lines on the microscope slide

Figure 28 shows a model picture of a biogas fermentor sample with a mixed microbial population. It was distributed on a microscope slide. Normally, 20 images per a sample are taken in a line and counted, in order to increase statistical assurance. However, it was interesting to assess what the middle value from 20 pictures per each acquisition line (1 top line, 2 between middle and top line, 3 middle line, 4 between bottom and middle line, 5 bottom line) would deliver (see figure 29). The middle value has to be a representative for cell counts in the entire area of the sample. The best acquisition line was looked for.
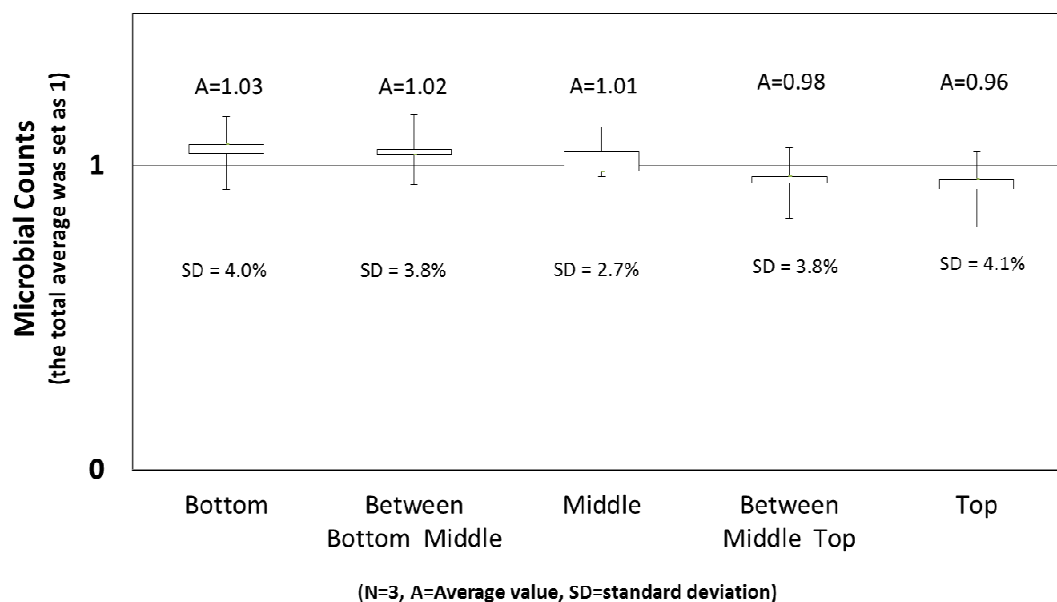
Figure 29 Microbial counts of a mixed microbial population at 5 different image acquisition positions on a microscope slide (figure 28)

In order to determine the best position for image acquisition, five different horizontal lines were chosen. At each line, approximately 20 pictures were taken and the microbes on these pictures were counted. On the y-axis, the total average value of microbial counts were set as 1. This procedure was repeated three times (N=3). About 2.7–4.1% deviation of the middle value of three repetitions was observed. The lowest value of the standard deviation was found in the middle line of the sample area. Interestingly, the mean value of the middle line (1.01) was also the nearest value to the total mean value of 1. Thus, the acquisition of images normally started on the left corner of the middle line in this study.

Effect of the dilution factor of a sample on the counting results

Cell density of a sample was also a factor that affected the cell count, which could vary by dilution. For instance, a low cell density of the distributed sample might cause a higher deviation of cell counting in different image acquisition positions (see figure 30).
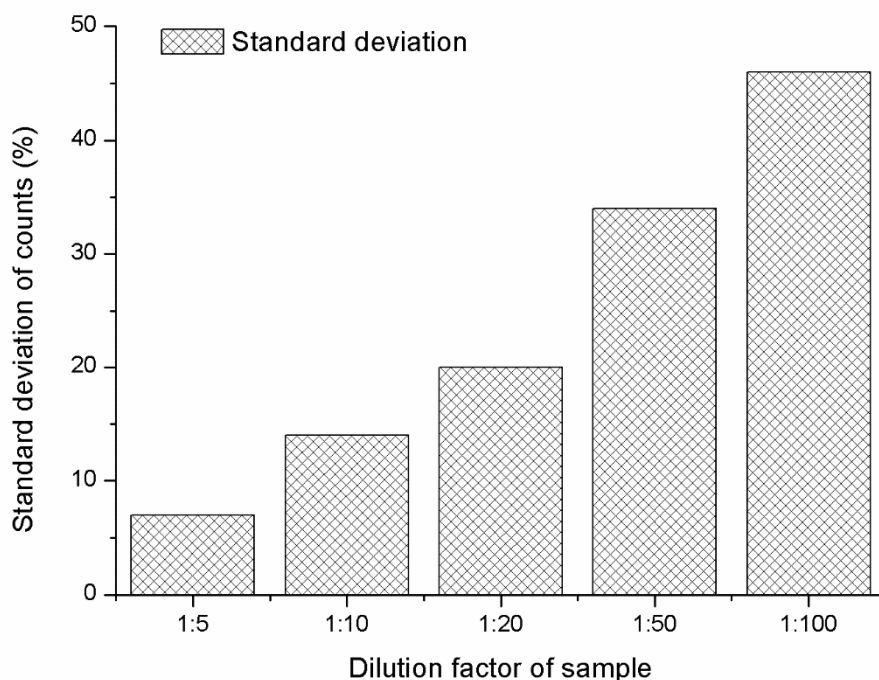
Figure 30 Effect of sample dilution factor on the standard deviation of counting results with fluo beads

Standard deviation of counts was measured depending on 5 different densities of fluo-beads distributed on the slide as a result of 5 different dilution factors. A solution of standard fluo-beads (4 μm, 4E+8/mL) was diluted by 5 different factors (1:5, 1:10, 1:20, 1:50 and 1:100). At each dilution, the fluo-beads were counted under 50x magnification at 5 different sample positions on the microscope slide (left, right, above, bottom and middle). Depending on the dilution factor, the density of distribution was varied. At higher dilution factors, the density of beads was low (7 %) and the standard deviation of counts of the distributed beads was high (46 %). The deviation could also be caused by electrostatic charging of the tube induced by vortexing in the case of low cell density (Brando et al. 2001). Thus, the higher density of cells in an image should be pursued wherever possible, but objects should occur as a single form to be countable. This was the case for fluorescence beads with a relatively low concentration. For total cell counting, the sample was normally diluted 60 times (1:60) and for methanogens 30 times (1:30) in

the case of a high density samples e.g. > 2E+10/mL of total cells and > 8E+8/mL of methanogens. A sample with lower density was diluted 40 times and 20 times for total cell counting and methanogenic cell counts, respectively.

Optimal concentration of stains for cell counts

Optimal concentration of SYBR Green I for staining bacteria was assessed. Monoculture of *E. coli* (3.3 E+8/mL) was stained with five different concentrations of SYBR Green I and the staining grade (%) was checked (figure 31).
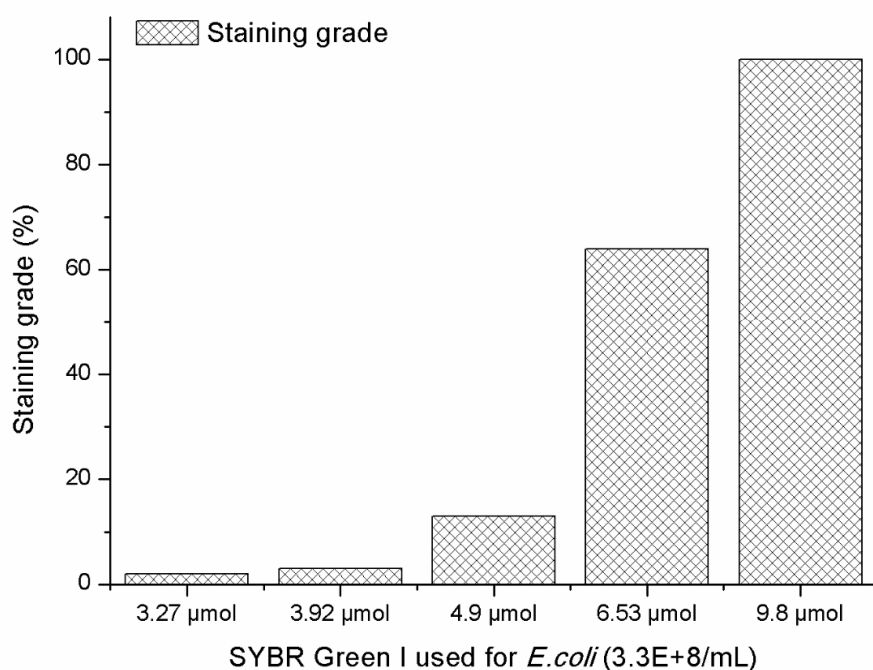
Figure 31 Optimal concentration of SYBR Green I to stain 100% of microbial cells

Optimal concentration of SYBR Green I was found at 9.8 µmol to have reached 100% staining of an *E. coli* cells of a mono-culture (DSM 498). Thus, 9.8 µmol of SYBR Green I was used as final concentration for all measurements, i.e. approximately 1000 fold diluted solution of the SYBR Green I stock solution (Zipper et al. 2004). SYBR Green I should bind DNA of all organisms, including bacteria and plants. However, the fluorescence intensity of plants was approximately 4 times less than that of bacteria tested in this study (results not shown). According to the different fluorescence intensity as well as their size, fluorescent methanogens could be automatically differentiated from

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fluorescent plant-particles by the image analysis software (Gross et al. 2010). Staining grade may be dependent also on ion concentration and the pH value of the medium (Bölter et al. 2002). However, in this study, such an aspect was not systematically investigated. In general, 9.8 µmol of SYBR Green I was sufficient to achieve 100% staining grade of cells as proved for more than several hundred measurements of various biogas plants samples. However, rod-type methanogens, mostly referring to *Methanobacteriales,* were poorly or not stained by SYBR Green I, while coccoid-type methanogens like *Methanomicrobiales* as well as aceticlastic methanogens were very quickly stained. The reason for this might be the different cell walls and lipid membrane structure of rod-type methanogens containing a dense pseudomurein which only occurs in two orders of methanogens: *Methanobacteriales* and *Methanopyrales* (Jarrell and Hamilton 1985; Valentine 2007; Visweswaran et al. 2010). However, the quotient of rod-type methanogens to total cells would be <10% under thermophilic conditions, and <5% under mesophilic conditions according to our experience. Thus, it should be taken account for total cell counts in the case of thermophilic conditions where rod-type methanogens are dominant. The staining process was done on the microscopic coverslip by pipetting and took 5-10 seconds.

Fluorescence microscopy should be performed promptly because microorganisms lose fluorescence very quickly (1–10 seconds depending on the species) during image capturing. Generally, coccoid-type methanogens showed about 1.3–2 times stronger fluorescence intensity than rod-type methanogens, but normally lost their fluorescence faster. Some species of coccoid-type methanogens (presumably *Methanoculleus*) were lysed (cracked) very quickly at a strong excitation of the fluorescence light. For this reason, the anti-fading agent DAPCO was always applied, see section 2.1.1. (Johnson et al. 1982), which significantly improved the fluorescing time of microorganisms.

Counting the methanogens: Methanosarcina and Methanosaeta

To count the number of methanogens (N/mL), an automatic counting method was realised by the image analysis software Image Pro 7. However, counting multi-cellular methanogens like *Methanosarcina* (packet-type aggregate) and *Methanosaeta* (sheathed filament-type) was difficult due to the three-dimensional aggregates and multiple overlapping cells by the automated counting method, although some image analysis methods tried to solve such problems (Blackburn et al. 1998; Zeder et al. 2010)
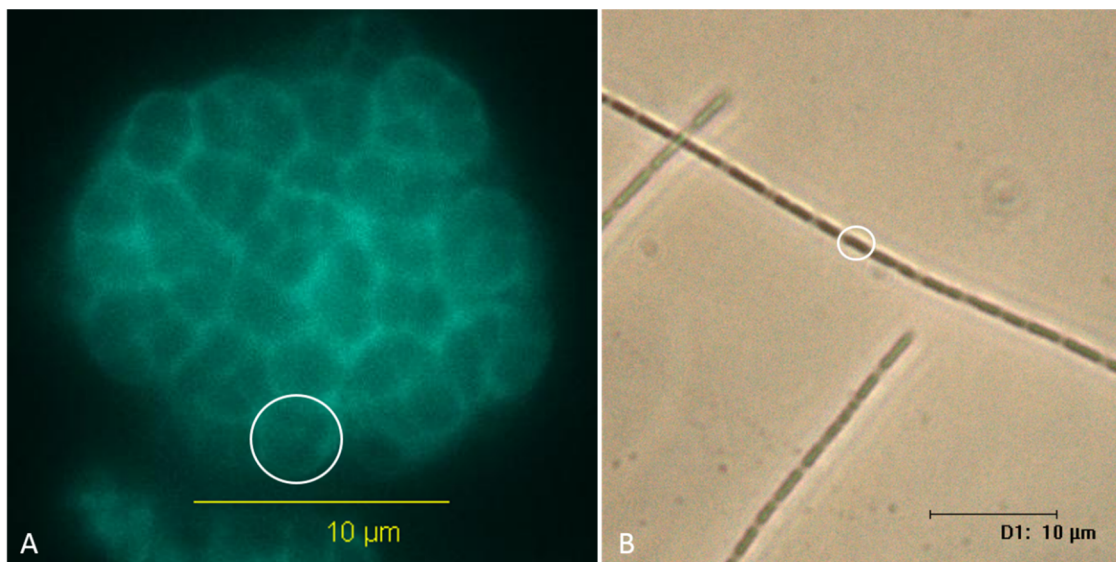
Figure 32 Example picture of *Methanosarcina* (A, cell aggregate) and *Methanosaeta* (B, sheathed filament cells)

Methanosarcina and *Methanosaeta* can be recognised according to their typical morphotypes (Boone et al. 1993). There could be other similar multicellular packets, e.g. *Desulphosarcina variabilis,* in anaerobic conditions, but these can be distinguished from *Methanosarcina* due to the methanogenic typical autofluorescence at wavelength of 420 nm. *Methanosaeta* occurs as a sheathed filament (long chain up to 300 μm, Hulshoff Pol et al. 2004) but has a very weak fluorescence. The F_{420} content of *Methanosaeta* was measured as 0.55 μg per mg protein (Zehnder et al, 1980). It was 2.8-times lower than the F_{420} content of hydrogenotrophs, which was about 2000 nmol per g protein (1.54 μg/mg protein, M F_{420}= 769.6, Reynols and Colleran 1987). Thus, it was difficult to differentiate between filament type bacteria and *Methanosaeta* according to the methanogenic autofluorescence. Thus, *Methanosaeta* should be confirmed additionally by molecular analysis. *Methanosaeta* was stained by SYBR Green I to be clearly observed in this study. Some *Actinobacteria* possess F_{420} (Selengut and Haft 2010) and can grow in anaerobic digesters (Wintzingerode et al. 2001), but the concentration was about 4.6–34 ng/mg protein, which is much lower than that of methanogens (Purwantini and daniels 1996). *Corynebacterium glutamicum* (DSM 20598) as one of the *Actinobacteria* was tested in this study and observed by fluorescence microscopy in our laboratory. It could not been detected at the excitation wavelength of 420 nm. The detection of *Methanosarcina* and *Methanosaeta* was performed manually.

Methanosarcina generally showed strong autofluorescence intensity. Their entire area and radius could be measured by the image analysis software. *Methanosarcina* generally occurred as multicellular packets in three dimensions. But they could occur as a single

cell depending on calcium and magnesium content (Boone and Mah 1987). For calculation of the number of *Methanosarcina* (figure 32, A, subunit in a white circle) and *Methanosaeta* (figure 32, B, single cell in a white circle), it was assumed as a model that *Methanosarcina* would consist of many subunits possessing a diameter of 2.5 μm and would occur as a sphere. For *Methanosaeta,* it was assumed that the length of a single cell of *Methanosaeta* would be 2 μm.

The number of subunits for *Methanosarcina* was calculated using the following sphere equation:

$$\frac{Volume\_of\_Whole\_Methanosarcina}{Volume\_of\_Subunit} = \frac{\frac{4}{3}*Area*Radius}{\frac{1}{6}*\pi*Diameter^3}$$

Equation 1 Calculation of the number of subunits of a cell aggregate of *Methanosarcina*

The calculated subunits of equation 1 were taken as number of *Methanosarcina* cells. Here, the diameter of the model subunit was 2.5 μm and π is a constant for a circle (3.14159). The area and radius of the whole *Methanosarcina* was measured by the used image analysis software.

The number of single cells for *Methanosaeta* was calculated using the equation:

$$\frac{Length\_of\_Whole\_Methanosaeta}{Length\_of\_Single\_Cell}$$

Equation 2 Calculation of the number of single cells of *Methanosaeta*

The length of a single cell was assumed to be 2 μm as a model. The whole length of *Methanosaeta* was measured using the image analysis software.

Generally, *Methanosaeta* was not (or rarely) found in agricultural biogas plants containing high solid content more than 5% (as well as high ammonium concentrations > 2000 mg/L) with long hydraulic retention time (> 60 days) by own experiences (results not shown). *Methanosarcina* has also been seldom found in agricultural biogas plants fed with energy crops like maize or grass without manure (by own observations, results not shown).

Detachment of Methanosaeta and Methanosarcina, without damaging

This remains a challenge to be solved in the future. Mild ultra-sonication could work, but there is a risk of damaging cells in the way that mechanical treatment does. As mentioned above, *Methanosaeta* was generally not found in agricultural biogas plants (Nettmann et al. 2010; Wirth et al. 2012), thus, the problem of detachment or differentiation of *Methanosaeta* was not the topic in this study.

Calculation of the size of sample area on the methylcellulose coated microscope slides

The three-dimensional liquid sample had to be transferred and distributed onto the two-dimensional sample on the microscope slide. The spread sample area must be known for the calculation of cell counts.
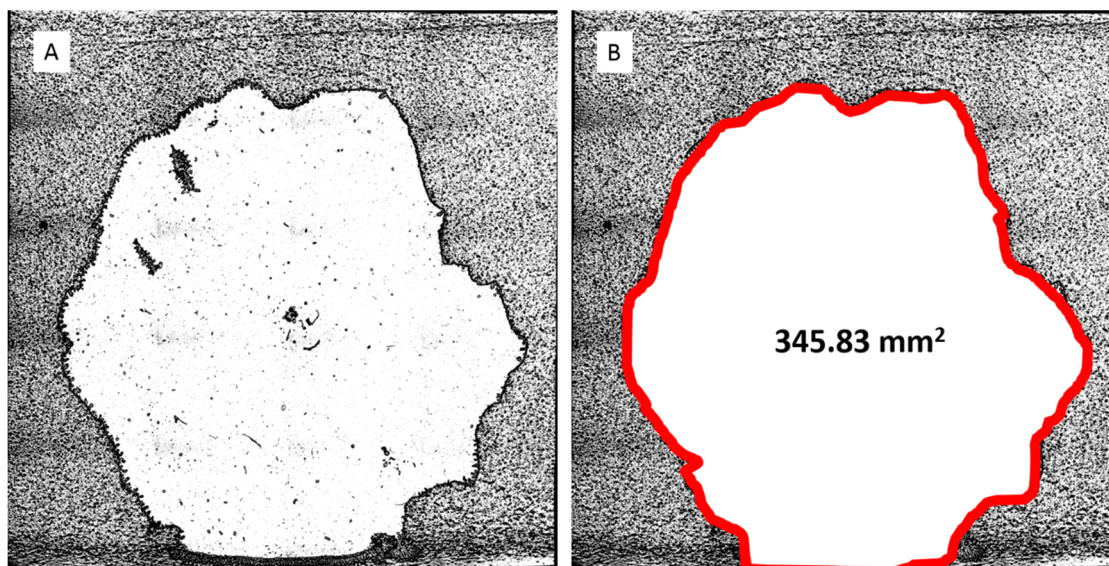
Figure 33 Calculation of sample area of a spread liquid sample under the coverslip on a microscope slide: Spread sample area on the methylcellulose-coated slide (A), calculated area (B)

10 µL of sample was distributed on the microscope slide. The sample area was calculated by a histogram-based quantification of the Leica software LAS AF after capturing about 20 images by a 1.25x loupe and merging the tile scanned images.

Calculation of cell number by image analysis

When all necessary measurement data were collected, including mean value of cells per image, area of sample, dilution factor and area of frame (image), cell counts were calculated with the following equation:

$$\frac{Cells}{ml} = \frac{Mean\_Value\_of\_Cells * Area\_of\_Sample * Dilution\_Factor}{Area\_of\_Frame * pipetted\_Volume * Conversion Factor}$$

Equation 3 Quantification of cell counts by microscopic image analysis

Example calculation: Mean value of cells in images = 498, area of sample distributed under coverslip on the microscope slide = 280 mm^2, dilution factor = 30, area of frame (image) = 0.03766098 mm^2, pipetted volume = 10 µL, and conversion factor from µL to mL = 0.001. As a result, (498*280*30) / (0.03766*10*0.001) = 1.11E+10/mL.

3.2. Validation of the developed technique to quantify microbial living cells by image analysis

The established microscopic image analysis technique should be validated with a microscopic reference cell counting method. Three different reference objects (4 µm artificial fluorescence beads, coccoid-type *Staphylococcus* DSMZ 6179, rod-type *E. coli* M15 DZ291, Qiagen) were counted using the developed microscopic image analysis technique and manual counting with a Neubauer counting chamber (Bast 1999).
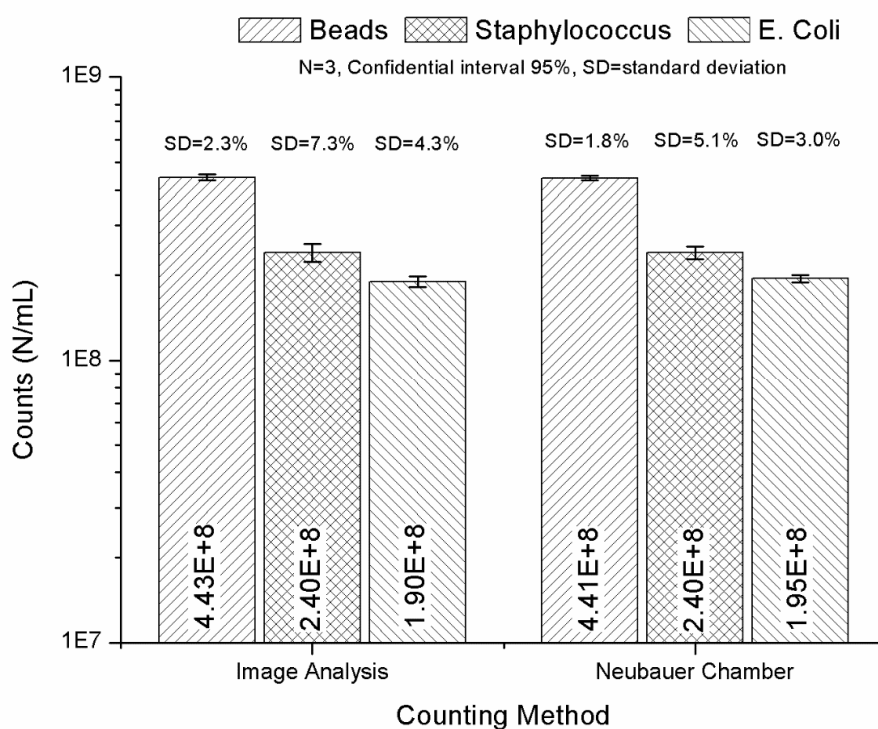
Figure 34 Comparison of two counting methods: Automated image analysis and manual Neubauer counting chamber

Three morphologically different types were counted by microscopic image analysis and manually by a Neubauer counting chamber (figure 34). The mean values of the counted objects were shown in the bar, respectively. An excellent congruence between image analysis (left) and counting chamber (right) was observed. The deviations between the two different counting methods, image analysis and Neubauer counting chamber, regarding fluo-beads, *Staphylococcus* and *E. coli* were very small, i.e. -0.2%, 0.3% and 2.6%, respectively. For each case, the standard deviation varied between 2.3% and 7.3% among three repetitions, while manual counting showed a slightly lower standard

deviation of 1.8%-5.1%. However, the values were generally acceptable when considering a very high number of objects up to 5E+8. The lowest standard deviation (2.3%) was shown for fluo-beads and the highest (7.3%) for *Staphylococcus* using image analysis. The reason for this may have been that the fluo-beads were very homogenous, with a regular shape and size. But *Staphylococcus* sometimes formed multi-clusters, although it was strongly mixed by a vortex so that the standard deviation was relatively high.

The test above referred to counting of monoculture without interfering particles. Another desired capacity of the image analysis software was differentiation between target objects and non-target objects depending on a different morphology automatically. The non-target particles could be plant particles, debris and soil particles interfering with accurate counting of target objects. Interfering particles can be divided into two parts: stainable particles like plant debris and non-stainable particles like soil particles. For the test, chemical ZnO nanoparticles were chosen as non-stainable particles and maize powder as stainable particles. Stained *E. coli* cells were counted as target-objects in the presence of the interfering particles. Image analysis software should count only the target objects according to their distinct morphology. To prove accuracy of counting *E. coli* with ZnO, the sample was counted in the phase contrast mode where both *E. coli* and ZnO could be seen. These were counted again in the fluorescence mode where only the stained *E. coli* could be seen, i.e. it was countable. Additionally, to prove accuracy for counting *E. coli* with stainable particles of maize powder, the sample was counted in the phase contrast mode where both *E. coli* and maize powder could be seen and counted again in the fluorescence mode where both *E. coli* and maize powder could be seen.
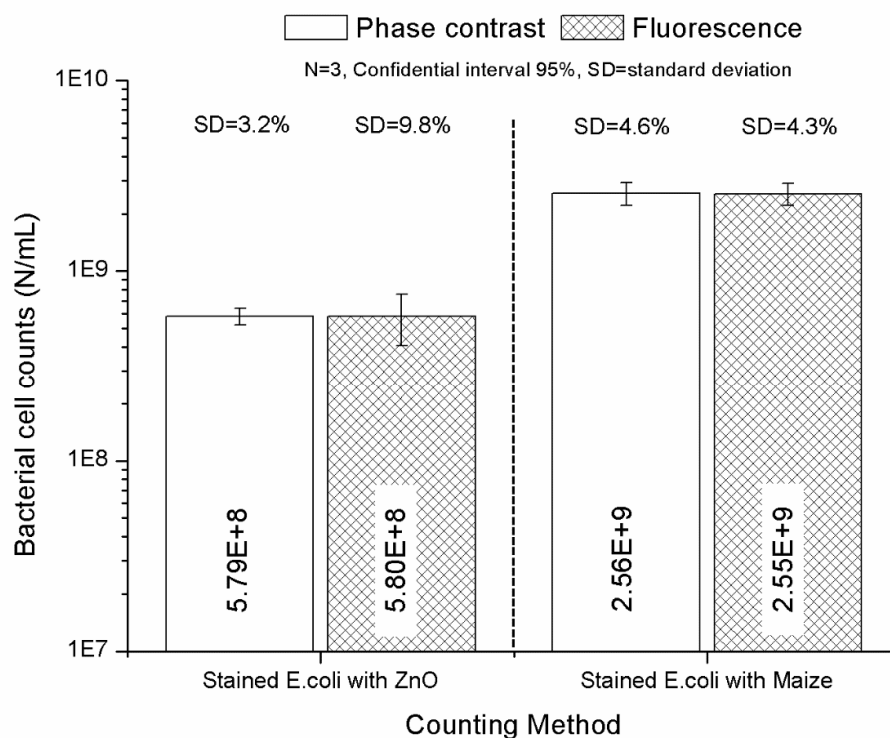
Figure 35 Counting *E.coli* in presence of interfering particles in phase contrast and fluorescence mode of the microscope

Figure 35 shows the results of testing the differentiating capacity of the image analysis in the presence of interfering particles (ZnO particles, maize powder). Zinc oxide was measured between 3 and 40 μm (NanoGard, 44898, Alfa Aesar Karlsruhe Germany). Maize silage (dry weight 10.4%) was dried at 105°C for 10 hours and micro-milled by a ball mill until the size of their particles was an average of approx. 4–40 μm. Irrespective of whether or not non-stainable or stainable particles were present, the image analysis counted the target objects correctly in the phase contrast mode as well as in the fluorescence mode. Counting results of target objects (*E. coli*) was almost identical between the phase contrast mode and the fluorescence mode. However, the standard deviation of counts with ZnO in the fluorescence mode was relatively high: 9.8% among three repetitions of measurement. The reason for that was unclear, but might be that cell density was reduced due to the addition of ZnO in the experiment; this caused a relatively higher standard deviation.

3.3. Applications of the developed digital image analysis to quantify and classify microbes

The developed microscopic counting technique was applied in practical biogas plants to determine the relationships between operational parameters and microscopic analysis data regarding quantification and morphological classification.

Long-term investigation of microbial cell counts found in the agricultural biogas plant Seth
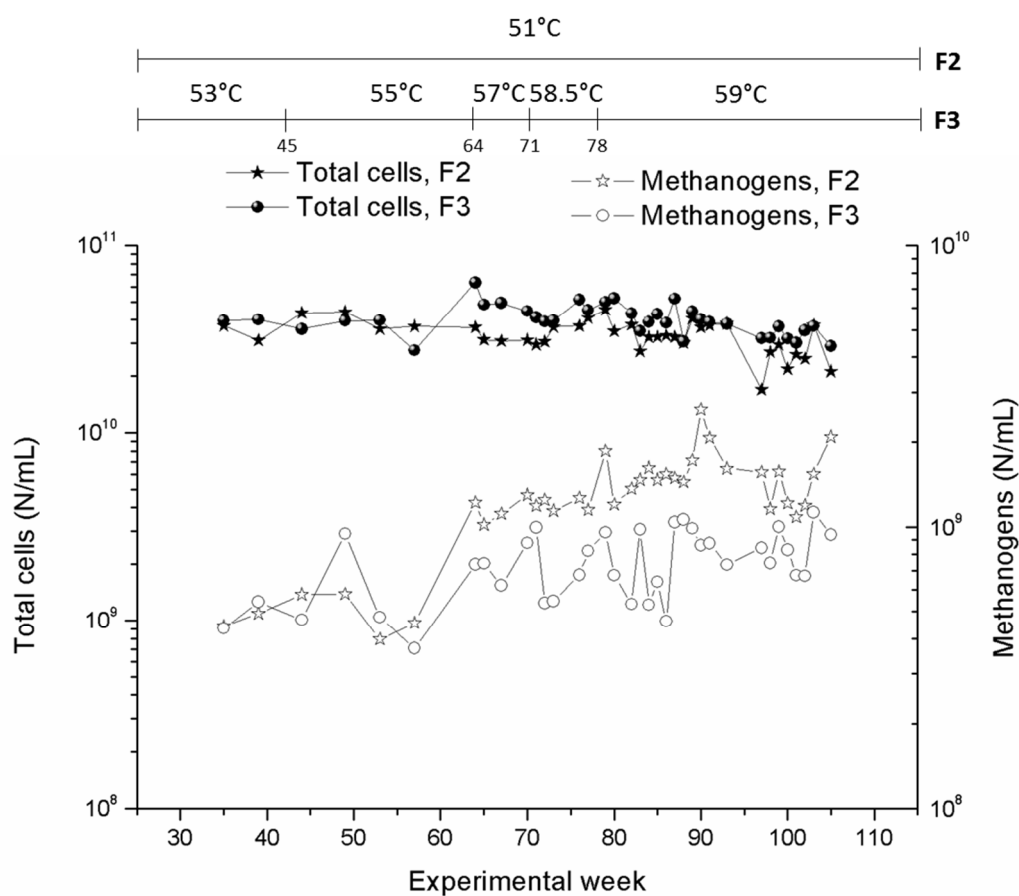
Figure 36 Long-term quantification of microbial cells in the large-scale biogas plant Seth

Long-term application of microscopic digital image analysis to count living microbial cells has been performed on a large scale German farmer biogas plant (location Seth) fed with cow manure (>70%), maize (<20%), grass (<10%) since July 2012, over more than 100 weeks. It was the first investigation on the microbial cell counts (N/mL) in a farmer biogas plant over a long period. The counts of total cells and methanogens in fermentor F2 and F3 were measured during the temperature increase of fermentor 3, while the temperature of the reference fermentor F2 was not changed. The fermenter F2 and F3 had

the same construction type and the same substrate feeding. The temperature of F3 was increased from 51°C to 59°C stepwise while the temperature of F2 was kept constant at 51°C to find the optimum temperature. In experimental week 57, the 50% maize input was replaced by grass for both fermentors, final feeding amount was the same as before. This caused a change in the microbial population after experimental week 57, interestingly in different ways. Apparently, by the increased temperature of F3, total cell counts were slightly higher that in F2, but not significantly. But regarding methanogenic cell counts, the two curves of F2 and F3 behaved apparently differently. The methanogenic cell number in the 51°C-digester F2 increased from 4.55 E+8 to 2.09 E+9, but from only 3.69 E+8 to 9.38 E+8 in the test digester F3. The methanogens of F2 were increased more (4.6 times) than those of F3 (2.5 times). Total cell counts ranged between 2E+10 and 8E+10 and methanogenic cell counts lay between 3E+8 and 1E+10. Interestingly, methanogenic cell counts varied more than total counts according to the change in temperature and substrate. The increase of methanogenic cell counts for both fermentors was initiated by the change in substrate from maize to grass. That also caused an increase in cobalt (results not shown). The further difference in the development of methanogenic cell counts between F2 and F3 was caused by temperature. Unfortunately, microbial quantification data regarding changes in feeding could not be found in literature. Another study reported a change in Archaeal community (qualitative analysis) regarding the increase in grass feeding, where the diversity of the Archaeal community was only slightly changed when the organic loading rate was increased by 40% (Wang et al. 2010). With the increased temperature of F3, the state of volatile fatty acids was relatively more unstable, than in F2, showing a propionic concentration up to 1400 mg/L between the 76th and 81st week, while a concentration of about half that occurred in F2 during the same period. However more interesting was the finding that at temperatures beyond 58°C, only rod-type methanogens could be seen which were identified as *Methanobacteriales* by qPCR (Kim et al. 2014). That means a low methanogenic diversity. Similar results were observed in another study (Krakat et al. 2010; Pervin et al. 2013). This might also be related to a lower stability and resistance to inhibitory events, like the accompanying accumulation of propionic concentration or the toxicity of NH_3. Another study compared the microbial community between mesophilic and thermophilic conditions and reported that thermophilic digestion was faster than mesophilic, but a about 5-fold higher VFA concentration (mg/L) and a higher population of cellulolytic microbes was reported than in mesophilic conditions (Shi et al. 2013). The higher VFA concentration in F3 could be related to a deficiency of trace element especially nickel. After addition of the nickel

containing trace element solution, the VFA concentration was dramatically decreased (results not shown), while there was apparently no effect when adding a trace element solution without nickel.

Monitoring of morphological changes of methanogens as a new microbial indicator for ammonia perturbation

To the best of our knowledge, morphological changes of methanogens in anaerobic digesters have not been investigated systematically to date, in terms of operational parameters. Using the same fermentors, F2 and F3, a very interesting phenomenon was found (figure 37).
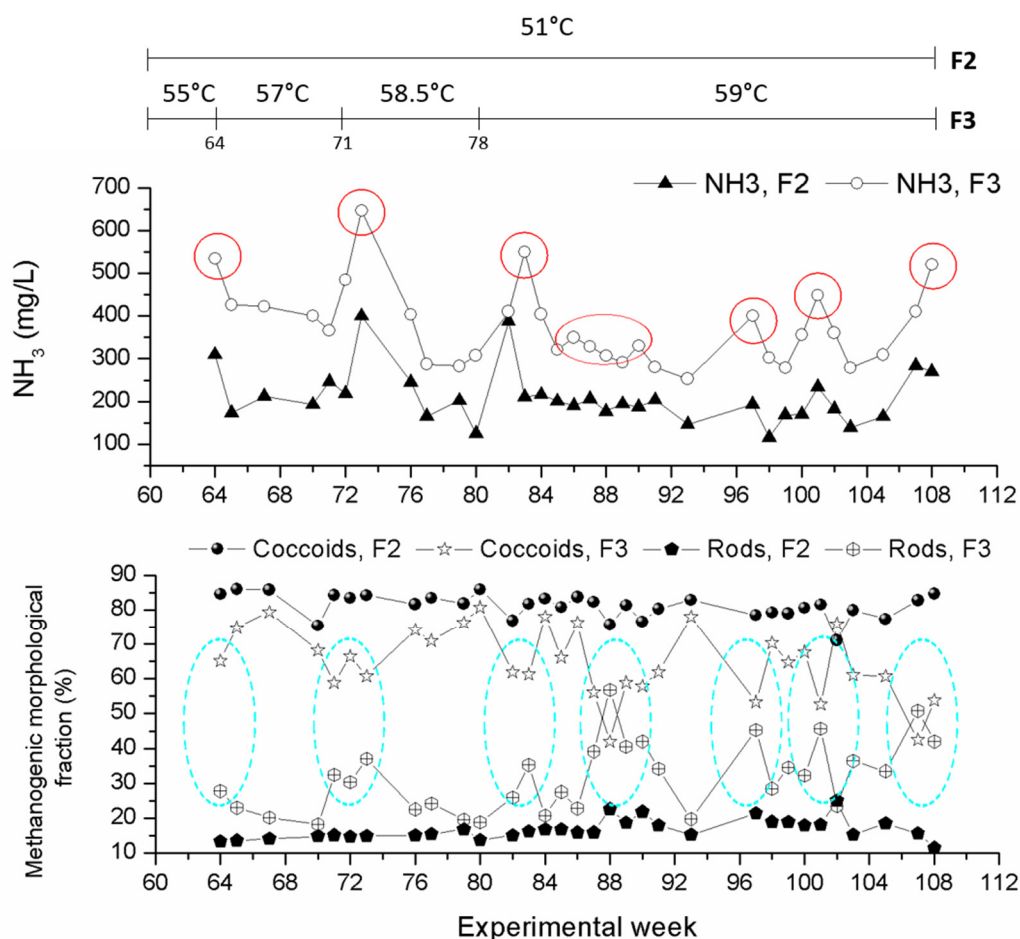
Figure 37 Monitoring of methanogenic morphological changes depending on ammonia concentration in the thermophilic full-scale biogas plant Seth

The ammonium concentration (NH_4-N) of both fermentors was measured by a spectrometer (Spectroquant, Merck, Darmstadt), varying between 1400 and 2300 mg/L. This value was calculated for ammonia concentration (NH_3-N) depending on pH and

temperature (Hansen et al. 1998). NH$_3$-N has been recognised as the main cause of growth inhibition due to its high permeability through the membrane (Gallert et al., 1998; Rajagopal et al., 2014). Several peaks of ammonia concentration occurred during fermentation, but values about two times higher were generally present in F3 (55-59 °C) than in F2 (51°C), mainly due to the increased temperature. The mean pH value in F2 and F3 during the experimental period was 7.6 and 7.7, respectively. The several peaks of ammonia concentration during 44 weeks of fermentation were apparently caused by the regular repair of an extra pump for cow manure, but not by other factors like substrate change or temperature increase. During this experimental period, the methanogenic morphological fractions like rod-type and coccoid-type methanogens were routinely observed and quantified by digital image analysis. Interestingly, a repeating behaviour of morphological changes was only found in F3, which seemed to be an exact reaction to the ammonia peaks. This morphological change was related neither to substrate change nor to temperature change. Volatile fatty acids (C$_1$-C$_5$) were also measured during the experimental period; but there was no clear correlation between the VFA-level and morphological change of methanogens. At that time, a change in gas production as a result of ammonia inhibition or accumulation of volatile fatty acids were not observed. Nevertheless, this phenomenon does not mean a direct indicator for ammonia perturbation but an observation which can be related to the ammonia perturbation. Hence, it was first suggested that this morphological change of methanogens might be used as an early warning microbial indicator for ammonia perturbation. Morphological change of microorganisms becoming smaller was reported e.g. in starvation conditions (Amy and Morita 1982; Roslev and King 1994), but this case can be excluded due to daily feeding to the fermentors in this study. It could be very helpful to find out such indicators before breaking down the bioprocess because the disturbance of fermentation by ammonia inhibition could be bound to serious economic losses (Sung and Liu, 2003; Calli et al. 2005; Nielsen and Angelidaki, 2008). The rod-type and coccoid-type methanogens were identified as *Methanobacteriales* and *Methanomicrobiales* by qPCR (Kim et al. 2014). At a higher ammonia concentration, the morphological fraction of the rod-type methanogens was increased and coccoid-type methanogens were simultaneously decreased. This phenomenon might be related to their membrane composition (see section 3.1. 'stains for cell counts'). The same relationship was also observed during analysing 29 different full-scale anaerobic digesters by De Vrieze at al., 2015. Therefore, it was assumed that rod-type methanogens might be more robust to ammonia inhibition or other

inhibiting circumstances. In order to prove this assumption, the following tests were conducted (figures 38 and 39).

N=15, Confidential interval 90%
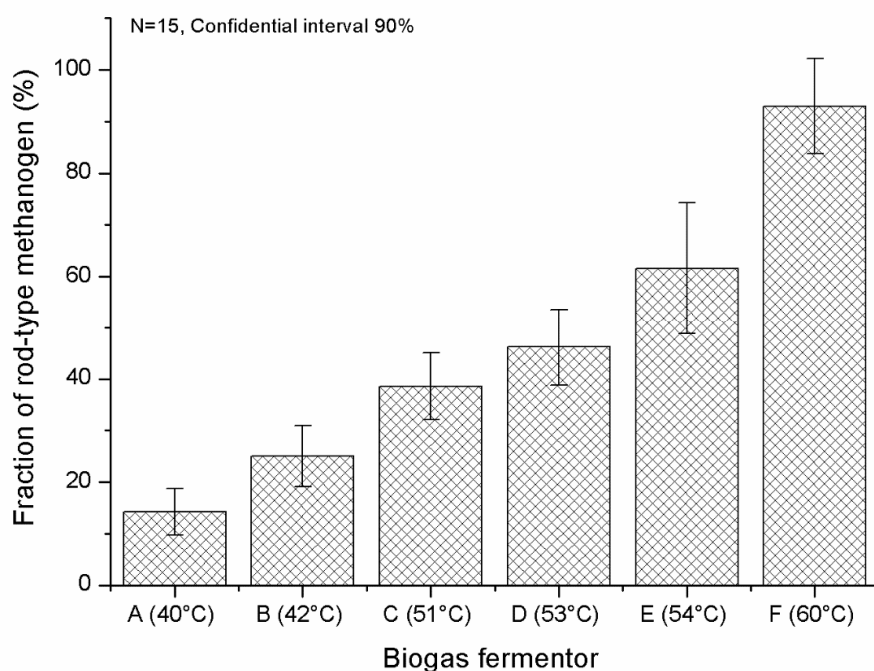
Figure 38 Effect of temperature on methanogenic morphology as seen by comparison of quantitative enumeration of rod-type methanogens in different biogas plants

The rod-type methanogenic fraction was measured in six different fermentors (A-F), which had six different operational temperatures: 40°C, 42°C, 51°C, 53°C, 54°C and 60°C, respectively. In this comparison, the substrate varied (A: Schuby fed with sugar beet, B: Hohenwart fed with grass, C: Niederau fed with sugar beet, D: Seth fed with cattle manure + maize, E: Viersen fed with maize + pig manure and F: RM60 fed with fodder beet). All fermentors were large-scale biogas plants in Germany, except RM60 which was a lab-scale reactor at our laboratory. At each sample, about 15 pictures (N = 15) were taken and the mean value was calculated. The fraction of rod-type methanogens increased with increasing temperature. Unfortunately, ammonia values and VFA data were not measured and collected in parallel, which might be proportionally related to the increase in temperature. It was interesting to see that the shift of morphotypes of methanogens from coccoid-type to rod-type was related to the temperature.

Lv et al. (2013) reported similar results, whereby *Methanobacterium* was the most predominant methanogen in the thermophilic digesters (50, 55 and 60°C), while in the mesophilic digester, *Methanosarcina* was the most predominant. Krakat et al. (2010)

observed this phenomenon for morphological changes from coccoid-type methanogens of *Methanomicrobiales* to rod-type methanogens of *Methanobacteriales* by using the ARDRA and FISH techniques along with a temperature change between 55°C and 60°C.

Another example of morphological changes according to unfavourable conditions is shown in figure 39.
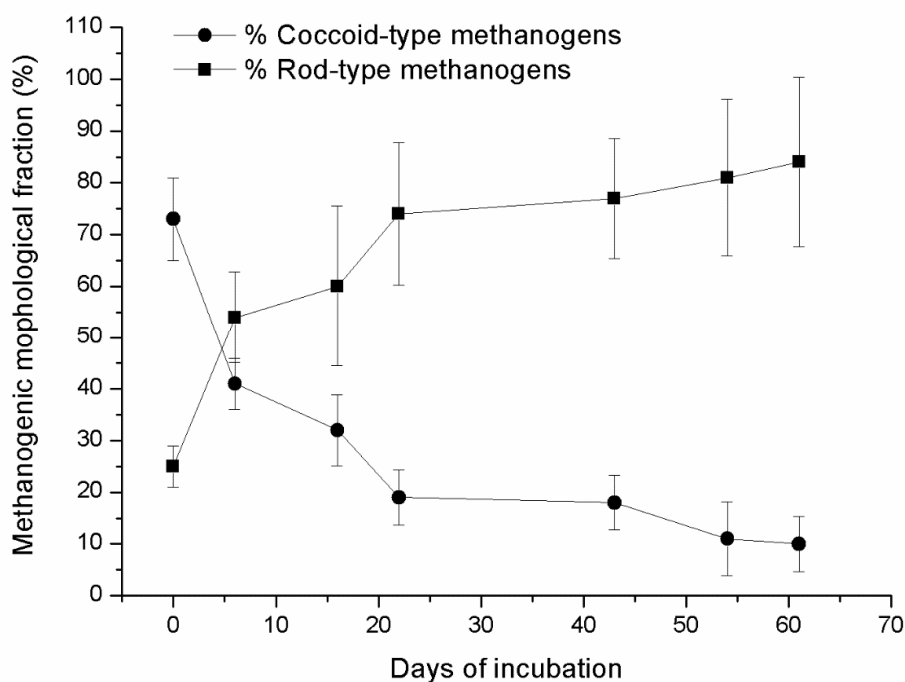
Figure 39 Effect of O$_2$ and starvation conditions caused a change from coccoid-type to rod-type methanogens

Archaea favour active maintenance by using energetic advantages in the form of reduced maintenance energy (Valentine 2007). A biogas plant Seth sample (cow manure + maize, total solid 8 %) was stored at 4°C in air for 61 days without substrate feeding (error bars: 90% confidential interval, N = 20 images). An aliquot of the sample was measured microscopically at seven different times. In the beginning, 73% of methanogenic coccoids were found, but they were reduced to 10% with time, while 25% of the methanogenic rods increased up to 84%. In this test, the rod-type methanogens seemed to be more robust to conditions such as starvation, which was in a good agreement with the observations of Cho et al. (2013) in the long term starvation condition during start-up of anaerobic digester treating swine waste water. It has been observed during long-term storage more than three weeks without feeding that rod-type and coccoid-type methanogens survived better than *Methanosarcina*. The *Methanosarcina* lost their fluorescence as well as their

structure of multicellular packets. The multicellular *Methanosarcina* were generally 100 – 3000 times bigger (v/v) than coccoids being 0.5 – 1 μm^3 and 400 – 10000 times bigger than rods being 0.1 – 0.5 μm^3, measured in this study. *Methanosarcina* seemed to need more nutrients (energy) in order to obtain and keep their structure. Consequently, multicellular packet-type *Methanosarcina* might be used as a microbial indicator for the state of the nutrients, suggested in this study. *Methanosarcina* should be found only in a good state of nutrients. On the other hand, the rod-type methanogens possessing smaller volumes showed longer stability and survivability. The reason could be the different methanogenic pathway using proton pump to generate energy. This proton pump from the water might be enough to survive and keep the structure for the hydrogenotrophs.

In this study, the morphological change as a new operational indicator was confirmed and improved as below in table 6, which was already observed in a similar way (Scherer et al. 2005) and based on a more concretised work by Scherer (Scherer et al. 2012). This can be used as a methodological option for the monitoring of anaerobic digestion.

Table 6 Morphological classification for monitoring of anaerobic digesters (see text below)

Morphology	Typical species of methanogens
Coccoids	*Methanomicrobiales*
Rods (< 6 μm length)	*Methanobacteriales*
Rods (> 6 μm length)	*Methanospirillum* (belongs to *Methanomicrobiales*)
Multicellular packets	*Methanosarcinaceae*
Straight sheathed filaments	*Methanosaetaceae*

Monitoring of microbial populations in anaerobic digesters can be performed by the fluorescence microscopy-based method. The quantification of bacterial and methanogenic cells could provide useful information about their activity concerning hydrolysis as well as methanogenesis (Kubota et al. 2009: Munk et al. 2010). Furthermore, it was attempted to morphologically classify the methanogens since the morphological diversity of methanogens occurring in biogas plants seemed to be relatively simple. The morphological classification was simplified and assumed (Kim et al. 2014), as shown in table 6, according to Boone et al. (1993) and Whitman et al. (2001), as well as to the observations in our laboratory, which were verified by the FISH technique (Scherer et al. 2005; Scherer et al. 2009, Krakat et al. 2010; Scherer and Neumann 2013). The taxonomic groups *Methanomicrobiales, Methanobacteriales, Methanosaetaceae,* and

Methanosarcinaceae were usually found to cover more than 90% of *Archaea* as well as less than 5% *Methanococcales* (Nettmann et al. 2008; Rastogi et al. 2008; Wirth et al. 2012). *Methanomicrobiales* have been shown to occur in various types (regular or irregular) of coccoids in biogas plants. It was found that hydrogenotrophs were dominant in agricultural biogas plants with energy crops where a high ammonia concentration was usually observed (Schlüter et al. 2008; Nettmann et al. 2008; Krakat et al. 2010; Nettmann et al. 2010; Wirth et al. 2012). Thereby, a coccoid-type *Methanoculleus* played a dominant role. In such cases of dominance of hydrogenotrophs, it could be simplified and presumably categorised as the coccoid-type methanogens being assigned to *Methanomicrobiales* (MMB) and the rod-type methanogens assigned to *Methanobacteriales* (MBT). This assumption is a rough estimate to rapidly see e.g. what type of methanogenic pathway dominant is, hydrogenotrophic or aceticlastic. A rod-type of *Methanomicrobiales*, *Methanospirillum*, could be distinguished from the rod-type of *Methanobacteriales* due to the criterion of length (6 µm) (Boone et al. 1993; Whitman et al. 2001). The following types of methanogens could not be differentiated morphologically by the microscopic technique: A rod-type *Methanomicrobiales,* e.g. *Methanoregula boonei,* or a coccoid-type *Methanobacteriales,* e.g. *Methanosphaera stadtmaniae,* as well as a coccoid-type *Methanococcales,* e.g. *Methanococcus maripaludis.* These methanogens might occur but only at a small number (might be negligible) in biogas plants. Therefore, the microscopic technique has to be supported and verified finally by a molecular-based method. The morphotypes of methanogens can be frequently monitored by the simple microscopic technique. The microscopic quantification and classification could provide a rapid and reliable analysis of the microbial population, despite its inaccuracy in the classification compared to molecular-based methods. The detection of *Methanosaetaceae* by microscopic techniques and qPCR was identical. However, in the presence of *Methanosarcinales,* the morphological classification had to be supplemented by molecular-based qPCR as coccoid-type *Methanosarcina* could also occur (Boone and Mah 1987). Some results were published in FEMS microbiology letters (Kim et al. 2014).

3.4. Development of an index by quantitative microscopic fingerprinting (QMFI)

Chemical analysis has been widely used for monitoring of the biological process in various anaerobic digesters. Chemical estimations are well established and useful for rapid evaluation (Rozzi and Remigi 2004; Scherer 2007; Ward et al. 2008), but the results are often regarded as not being sufficient for an accurate description of the biological process. Thus, a biogas fermentor is often called a black box. One aim of this study was to identify a microbial indicator for the quality assessment of the black box (Riviére et al. 2009; Kothari et al. 2014). This technique should be intentionally established for easy and fast applications. For this purpose, the previously described microscopic technique including digital image analysis was further improved in order to determine a microbial index. Thereby, approximately 38 agricultural large-scale biogas plants in Italy were analysed.

Establishment of basic structure of the indicator by methanogenic cell counts, methanogenic cell size and methanogenic fluorescence intensity

The assumption made in this study was based on a master thesis of Shaikh Y. made in the same laboratory that a higher number of methanogens should be strongly correlated to higher biogas production. This thought was plausible and could be found by some other researchers (Tagawa et al. 2000; Kubota et al. 2009: Munk et al. 2010; Traversi et al. 2012). This was the starting point for the index. Furthermore, it was assumed that the methanogenic cell number might be influenced by the substrate. All digesters (38) analysed from Italy for the identification of a new microbial index were fed by maize (>80%) and triticale (<20%). They all were operated under mesophilic conditions. The mean value of maize feeding was 83% and triticale was 17%. Thus, they had almost the same conditions regarding the substrate and operational temperature. However, despite the similar composition of substrate, methanogenic cell number varied strongly between 5.43E+8/mL and 5.13E+9/mL, a difference that was approximately 10-fold (figure 40), which might result in a different output of biogas. Thus, methanogenic cell counts were not only related to substrate. The methanogenic cell counts seemed to be influenced by more complex operational parameters.
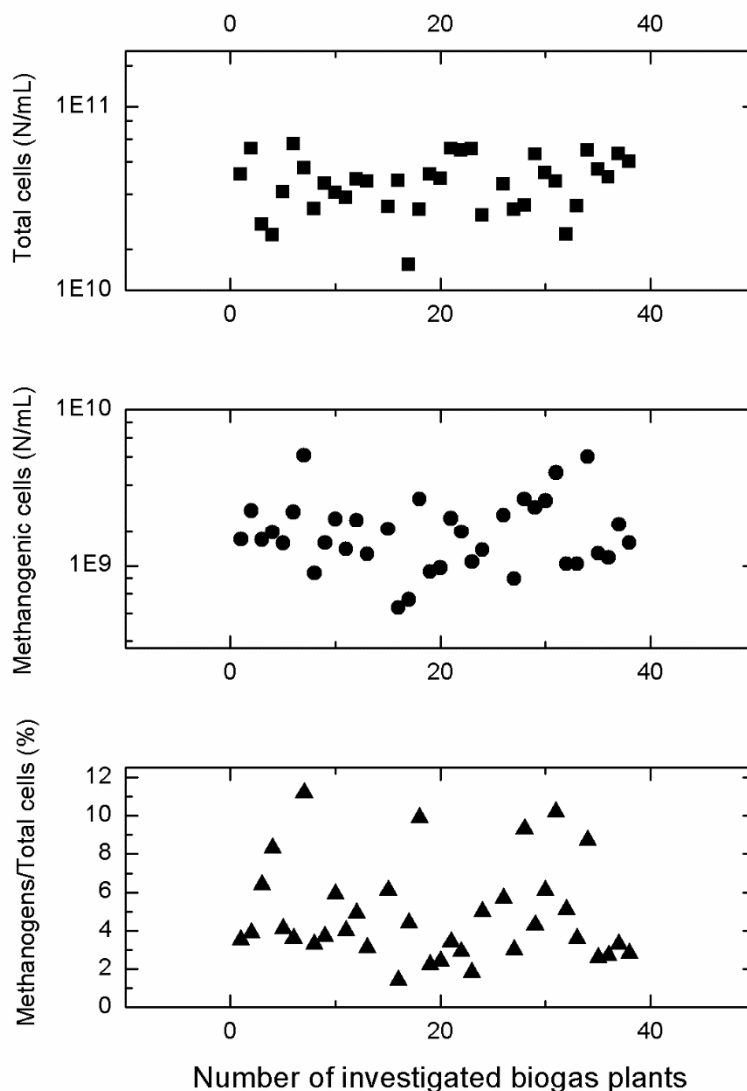
Figure 40 A series of 38 agricultural biogas plants from Italy was investigated for methanogenic cell counts, total cell counts, and their quotient, which were fed with 83% maize and 17% triticale

The mean value of methanogens was 1.88E+9/mL, but with a variation of approximately max. 10 fold. Based on total cell counts, the quotient of methanogens varied between 1.4% and 11.2% (mean value was 5%). During investigation, it was recognised that the methanogenic cell counts were dependent not only on their microbial activity, but also on process parameters like e.g. hydraulic retention time (HRT). Methanogenic cells grow relatively slowly and might be influenced significantly by HRT because of a wash-out effect. For example, the biogas plant Seth F3 had a HRT of 11 days and showed a relatively low amount of 8.34E+8 methanogens (quotient of methanogens to total cells was 2.2%). This fact could be caused by the short HRT, which might prohibit a higher

number of methanogens. The effect of HRT strongly depended on reactor type (Bialek et al. 2011; Schmidt et al. 2014). Thus, HRT was not concerned in this study due to its complexity. The methanogenic cell number was taken as a basis for the prediction of biogas production but it did not always correspond to methanogenic activity proportionally. Cells could have different activity states like stagnation, exponential growth, maintaining or dying (Pirt 1982; Valentine 2007).

In addition to methanogenic cell counts, another phenomenon was also observed, that all active methanogens were fluorescent based on methanogenic coenzyme F_{420} responsible for electron transfer (Cheeseman et al. 1972; Gorris and van der Drift 1994) and showed different intensities, which might be correlated to the level of hydrogen metabolism for methanogenesis (Deppenmeier et al. 1996). Furthermore, this fluorescence intensity should be proportionally related to the size of methanogenic cells (μm^2), where hydrogen metabolism occurred. If the capacity of coenzyme F_{420} was the same between species (or negligibly different), the larger cell size should have greater capacity containing the coenzyme F_{420} in cytoplasm for hydrogen metabolism than the smaller one. Both values of the fluorescence intensity and cell area could be measured by the microscope technique (figure 41). There were several papers about a positive relationship between methanogenic activity and F_{420} content (Eirich et al. 1979; Dolfing and Mulder 1985; Gorris et al. 1998; de Poorter et al. 2005), where the F_{420} content should have a positive relationship with the fluorescence intensity.
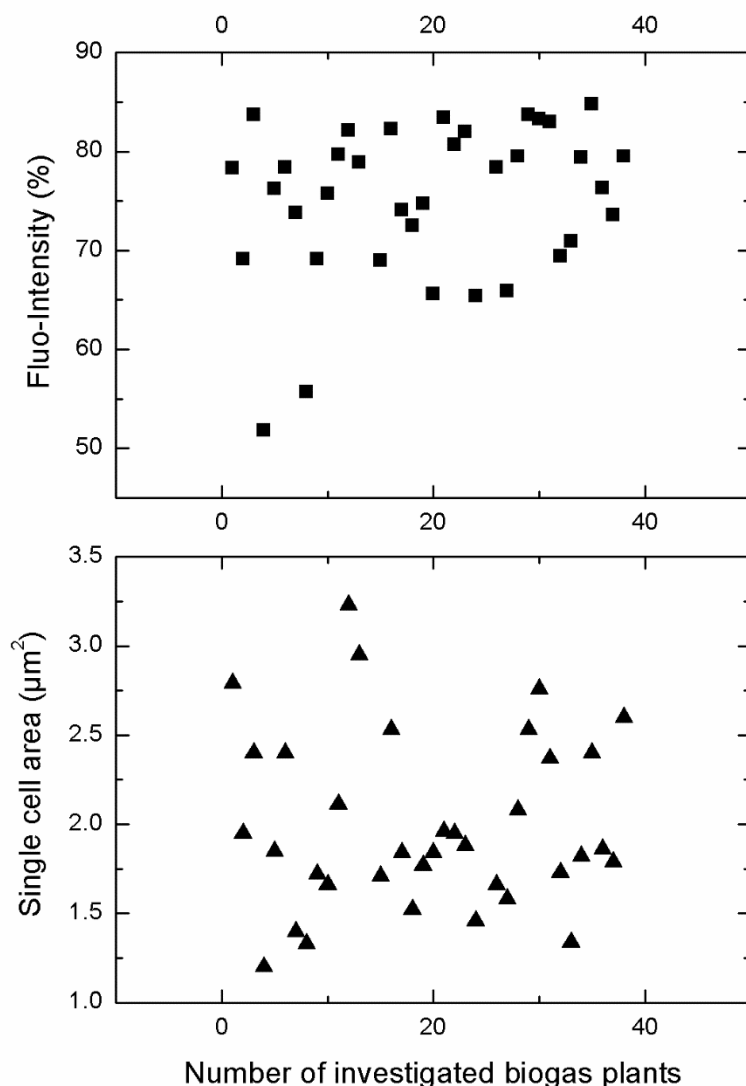
Figure 41 A series of 38 agricultural biogas plants from Italy was investigated for the fluorescence intensity and single cell area (The same series as in figure 40)

The fluorescence intensity of a series of biogas plants varied between 52% and 86% and their mean value was 76% (figure 41). The well degradable substrate maize combined with triticale normally showed a higher fluorescence intensity of methanogens compared to other substrates (figure 42). The mean value of the single cell area of a methanogen varied between 1.2 and 3.23 μm^2. Their mean value was 2 μm^2. Normally, coccoid-type methanogens like *Methanoculleus bourgensis* showed a cell area of 2.3-2.9 μm^2 while rod-type methanogens like *Methanothermobacter thermautotrophicus* showed a size of 1.4-1.7 μm^2 in this study. Coccoid-type methanogens are often 1.68 times bigger than rod-type methanogens and very often dominantly occurred in maize fed digesters producing a relatively high amount of biogas (Weiland 2010; Nettmann et al. 2010; Wirth

et al. 2012). For instance, a biogas plant with mono-coccoid-type methanogens showed a fluorescence intensity of 75% while a biogas plant with mono-rod-type methanogens had a fluorescence intensity of 42%. Thus, the fluorescence intensity should have a relevance to the size (morphology) of the methanogens. In other words, the size of the methanogens seemed to be relevant to methanogenic activity as well. Therefore, the size of methanogens should be included in the consideration of a factor for methanogenic activity in this study. However a clear correlation between cell size and fluorescence intensity of methanogens could not be seen in figure 41, i.e. which might mean a complex interaction between some influencing factors.
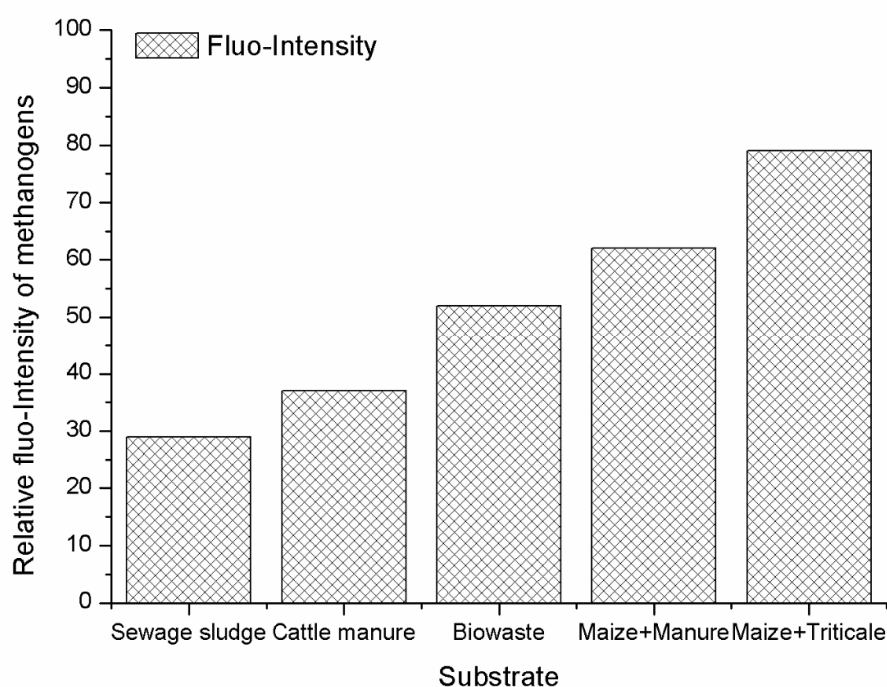
Figure 42 Variation of the fluorescence intensity of methanogens measured in samples of different types of biogas plants

Fluorescence intensity of methanogens on different substrates was investigated (figure 42). The substrates used were sewage sludge, cattle manure, biowaste, maize + manure, and maize + triticale as indicated in figure 42. The results of relative, microscopic fluorescence intensity of methanogens were 29%, 37%, 52%, 62%, and 79%, respectively. The order of substrates was proportionally related to the level of biogas yield of the substrate (Weiland 2010).

Three influencing factors for biogas yield (or methanogenic activity) were found as methanogenic cell counts, methanogenic cell size and methanogenic fluorescence intensity. However each factor did not necessarily be correlated each other. For instance, there could be found many methanogenic cells but they could not necessarily shine highly, i.e. they might be dying. Their size could be big but they could not absolutely have a higher fluorescence intensity. But the three factors can simultaneously influence the methanogenic activity. From those facts and measurements, a new term was consequently created and simplified as a product of methanogenic cell counts (N/mL) * mean value of a single cell area (μm^2) * mean value of fluorescence intensity (%) in a unit of [1/cm], which was assumed to describe the total methanogenic cell power (TMCP). This TMCP value is actually based on methanogenic cell counts (N/mL) as the main factor but involves two additional factors: the cell area (size) and the fluorescence intensity. The TMCP value is a measured value which might be used for the prediction of biogas activity. The TMCP of 38 digesters varied very strongly between 6.7 and 78.3, i.e. the difference of 11.7-fold. The mean value of the TMCP was 29.3.
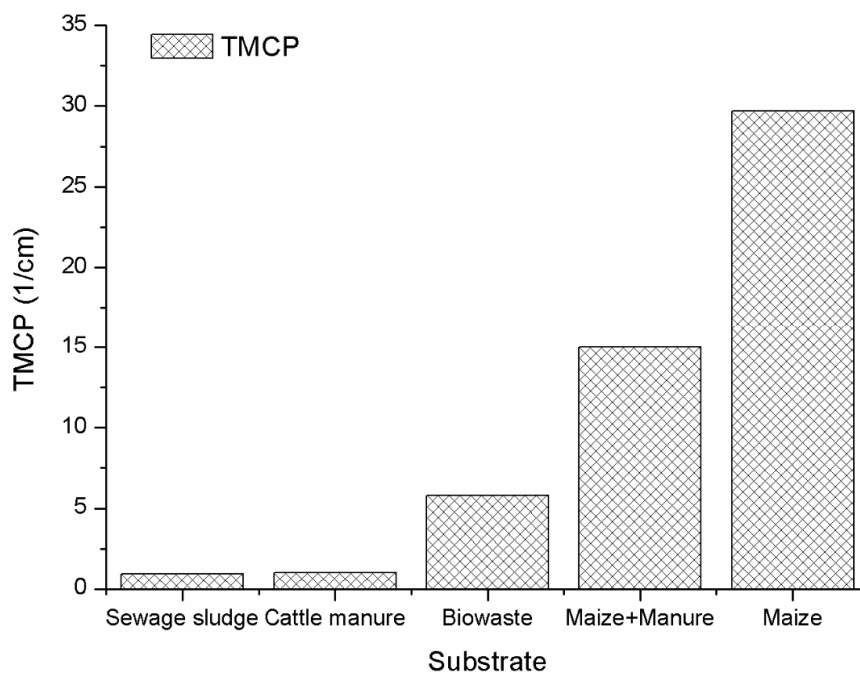
Figure 43 Demonstration of the variation of total methanogenic cell power (TMCP) depending on substrate in different biogas plants

Total methanogenic cell power (TMCP) was measured in five different plants with 5 different substrates as sewage sludge, cattle manure, biowaste, maize + manure, and

maize in figure 43. In the plant with maize, the highest TMCP value (29.7) was obtained while sewage sludge showed the lowest value (0.91) for the TMCP.

Addition of chemical habitat data to microscope data

A significant problem still remained when seeking a microbial index describing the quality of the bioprocess. For example, a biogas digester Parkstetten with fed maize and grass showed 15000 ppm of propionate and 22900 ppm of total volatile fatty acid (pH 7.6). The bioprocess was broken down. However, methanogens were still there and had a fluorescent intensity of 83.4%, which was very high. The methanogenic cell count was 3.97 E+8. Although biogas production was broken down, a number of methanogens were still active. For this case, methanogens were probably in the phase of maintenance, which could be understood in the same context as figure 25. The singularity of *Archaea* to chronic energetic stress is suggested to the adoption to the environment to reduce maintenance energy, where the ratio of energy requirements for survival, maintenance and growth of Archaea was estimated at $1:10^3:10^6$ (Valentine 2007). The TMCP of the disturbed process was 11, a relatively median value compared to other substrates (figure 43), meaning a median state of the total methanogenic activity. Although the TMCP indicated a median state of methanogenic activity of the disturbed biogas plant Parkstetten, propionate concentration was over 15000 ppm and biogas production was broken down. It did not fit well with the TMCP and was not clearly understood. Additionally, it was observed that some acidified biogas plants showed a number of active methanogens (results not shown). Hence, the TMCP did not seem to be sufficient to predict all situations of the biogas process. Some parameters may be missing in the equation of the TMCP. Consequently, this non-matching fact was one of the reasons for combining the microscopic results with chemical analyses of microbial intermediates like volatile fatty acid (VFA, mg/L), volatile solid (VS, mg/L), and degradation efficiency. These metabolic properties were added to the TMCP formula in order to match the microscopic results to the reality of the bioprocess in anaerobic digesters in 1/cm, as follows (Eq. 4):

$$\left(1 - \frac{VFA}{VS * Degradation\_efficiency}\right) * TMCP$$

Equation 4 Combination of the developed TMCP with metabolic products to improve the indicator

In order to improve the relation of the new microbial indicator to the methanogenic activity, the content of acetic acid should also be considered. Acetic acid is a key product of the entire fermentation process, being cleaved to methane acetoclastically or converted via syntrophic acetic acid oxidation to hydrogen and carbon dioxide, and further hydrogenotrophically to methane (Thauer 1998). A low concentration of acetic acid generally indicated a stable process having efficiently converted acetate to methane. It is well known that a too high acetic acid (HAc) content of the digester medium could mean a lower gas production rate, indicating that acetic acid was not completely degraded to methane gas i.e. inhibition of methanogenesis could have occurred (Fukusaki et al. 1990). The inhibition can occur by various reasons like a too high concentration of sulphide, ammonia, heavy metals, antibiotics, and trace element deficiency, by which acetic acid could consequently be accumulated (Chen et al. 2008). The poorly degradable propionic acid can also increase, which also shows a significant inhibitory effect on methanogenesis (Wang et al. 2009), but is mainly caused by overloading of substrate in our experience. In the case of the accumulation of propionic or butyric acid, the concentration of total volatile fatty acid values (VFA) becomes larger than the acetic acid concentration. In that case, total VFA values should be involved in the calculation of the new indicator, instead of acetic acid value. Otherwise, the concentration of acetic acid is normally similar to that of total VFA for a stable process. VFA concentration should be directly related to the degree of methanogenesis, which should be inversely proportional to the rate of methanogenesis. For generalising (normalising) this concept, VFA concentration was divided with the product of volatile solids and the degradation efficiency. The term of VFA concentration per (volatile solid * degradation efficiency) was involved to the equation and should be an important parameter for the prediction of the performance of a biogas fermentor. The term of (1-VFA/(VS*degradation_efficiency)) combined with the TMCP might be theoretically proportional to the biogas production rate. An approximately positive relationship between VFA concentration and VS*degradation_efficiency in 38 biogas plants is shown in figure 44.
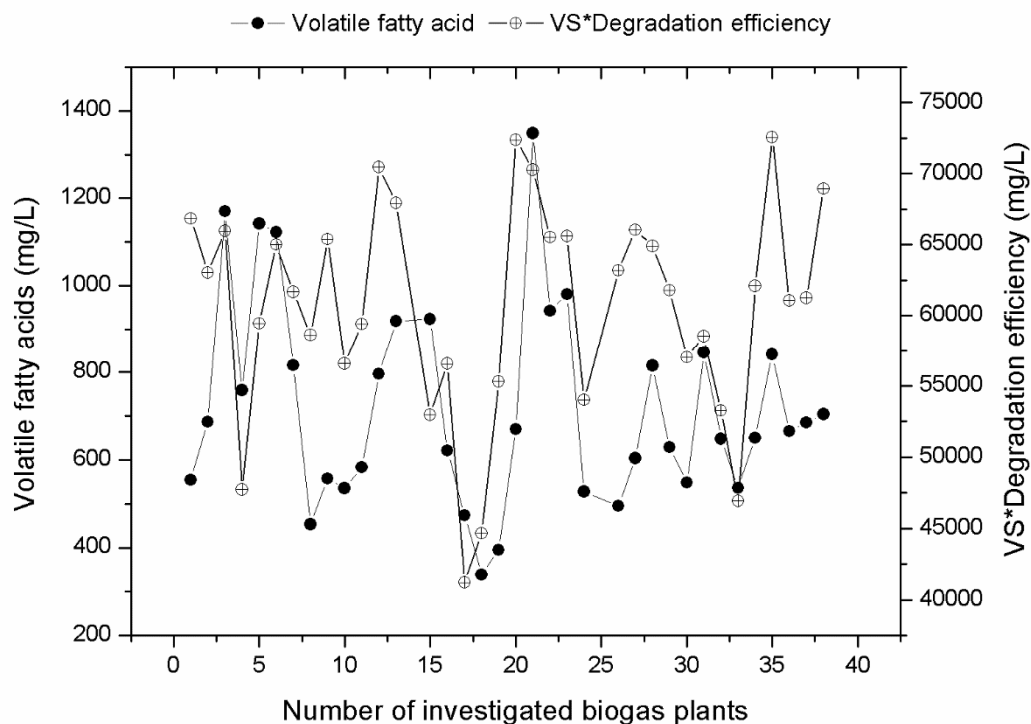
Figure 44 Correlation between volatile fatty acids produced and volatile solids*degradation efficiency of 38 agricultural biogas plants from Italy

Similar pattern was shown between volatile fatty acid concentration produced and the product of volatile solids and degradation efficiency as found for 38 biogas plants (Pearson correlation coefficient 0.47).

$$Degradation\_efficiency = \frac{\left(VS_{input} - VS_{output}\right)}{VS_{input}}$$

Equation 5 Simplified calculation of the biological degradation efficiency

Degradation efficiency (unit-less) was roughly calculated according to equation 5. VS stands for volatile solids. In this equation, lignin content was not involved, which is not degradable during anaerobic digestion. For more accurate calculation of degradation efficiency, the lignin content can be involved by subtracting the lignin content from each term of the equation 5, i.e. $(VS_{input} - VS_{output})/(VS_{input}-VS_{lignin})$. The lignin content is constant during digestion. The lignin content of different substrate can be found e.g. in a paper (Dandikas et al. 2014)

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Generalisation and fine-tuning of the equation for the indicator

In order to make the value of equation 4 more comparable for generalisation of the equation, which means that the values should vary e.g. between 0 and 1; the equation was transformed as the following formula (equation 6):

$$\frac{\left(1 - \dfrac{VFA}{VS * Degradation\_efficiency}\right) * TMCP}{A + \left(1 - \dfrac{VFA}{VS * Degradation\_efficiency}\right) * TMCP}$$

Equation 6 Generalizing the of values of equation 4 varying between o and 1

'A' refers to a regulating term which decreases the result of equation 6, in the manner of an inhibition factor. Equation 6 can be proportionally related to the output of a biogas process i.e. the biogas production rate. Equation 6 was mainly based on methanogenic cell counts, afterwards added by a metabolic product of VFA. In the context between methanogenic cell counts and VFA concentration, the regulating term (A) should be involved in equation 6, which had to relate to the methanogenic cell counts as well as to VFA concentration. Thus, a term of total cell counts based on (normalised) methanogenic cell counts was added which should regulate the value of equation 6. Microorganisms could use energy not only for growth but also for maintenance, especially in the case of acidified fermentors. Both cases could probably show higher microbial activity. It was often observed that highly acidified processes contain a higher number of fermentative and acidogenic bacteria compared to the number of methanogens (figure 45) in this study. It was similarly observed that a higher number of *Bacteroidetes* has been shown to correlate higher hydrolytic activity (Regueiro et al. 2012). Inversely, the quotient of total cells to methanogenic cell counts was generally low, varying between 5 and 10 at a stable biogas plant like digester A (Lanken) fed by maize, producing high amounts of biogas as well as a high number of methanogens, more than 3E+9 (figure 45).
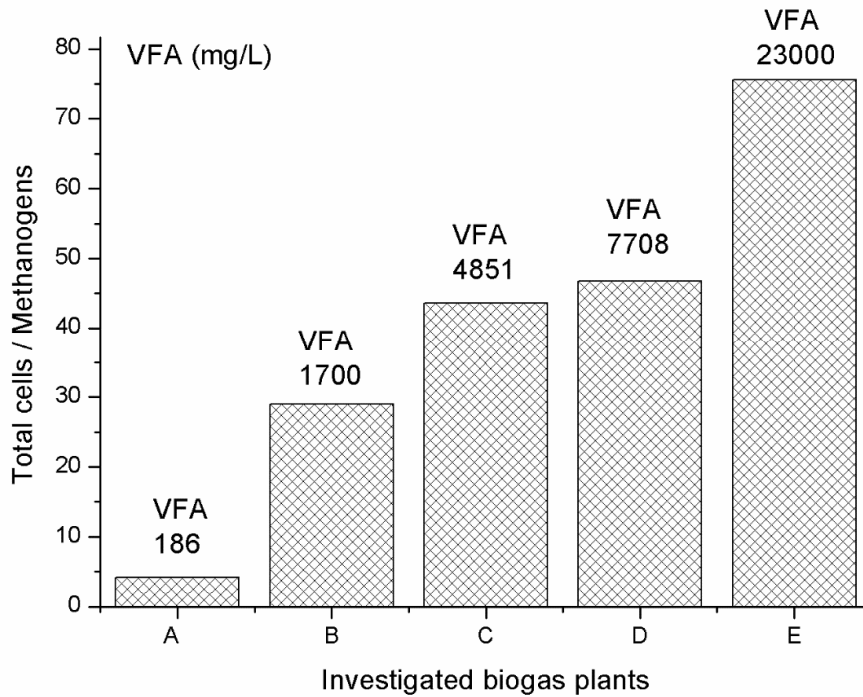
Figure 45 Quotient of total cells to methanogens in relation to various volatile fatty acid concentration

In figure 45, a proportional relationship between volatile fatty acid concentration and a quotient of total cells to methanogens in different fermentors with different operational situation (A: Lanken fed with mono maize under mesophilic, B: Seth F3 fed with manure + maize under thermophilic, C: Seth F3 fed with manure + maize under thermophilic, D: Seth F3 fed with manure + maize under thermophilic, E: Parkstetten fed with maize + grass under mesophilic condition) was shown. The quotient was proportionally increased from 5 to 75 with an increase in VFA concentration from 186 to 23000 mg/L.

Therefore, a term of the ratio of total cells to methanogens (total cells/methanogens) could replace the term 'A', the inhibition factor, which should negatively influence the indicator equation.

$$\frac{\left(1 - \dfrac{VFA}{VS * Degradation\_efficiency}\right) * TMCP}{\dfrac{TotalCells}{Methanogens} + \left(1 - \dfrac{VFA}{VS * Degradation\_efficiency}\right) * TMCP}$$

Equation 7 Insertion of a regulating term replacing the term A of the equation 6

To this point, the basic shape of the equation has been established. There is only an improvement of fineness of the equation left. The value of (1-

VFA/(VS*Degradation_efficiency)) was not sensitive at all. The VFA/(VS*Degradation efficiency) was approximately 1.2%. Therefore, the term VFA should be strengthened empirically.

Involvement of a concept "biodiversity" in-between microscopic data and chemical habitat data as a strengthening factor (Link between cell counts data and chemical habitat data)

There was another important term regarding biological stability and the capability of resistance against perturbation, i.e. biological diversity (Girvan et al. 2005). The term VFA/(VS*degradation efficiency) means the metabolic state of the bioprocess which should be connected to cell counts in order to show the level of microbial quality of the bioprocess. The concentration of VFA could work as a positive or negative interacting factor between cell counts and VFA concentration. In this context, the biological diversity could be a good candidate to be involved in this point to strengthen the term VFA/(VS*Degradation efficiency). The microbial diversity could be measured by a molecular analysis e.g. qPCR. However, the biodiversity could be measured using the microscope more simply and rapidly i.e. morphological biodiversity (Liu et al. 2001). VFA concentration always depends on volatile solids concentration (mainly plant particles and microorganisms), which mitigates and buffers but also utilises VFA simultaneously. It could be a stabilising factor (f_S) in relation to VFA. Thus, the term VFA/(VS*degradation_efficiency) should be strengthened together.

The question was, how it should be strengthened. Biodiversity could be used as a 'strengthening factor' which might be related to the stability of the bioprocess against inhibiting occurrences like the accumulation of VFA. It is generally known that greater species diversity could, on average, increase the stability of yield, resistance to inhibiting conditions, nutrient demineralisation and decrease disease prevalence, but also depends on the ability of the community or a functional group that might be dominant (McCann 2000; Girvan et al. 2005). An assumption was made that morphological biodiversity positively influences the stability of gas yield and resistance to inhibiting conditions which is in a good accordance with other studies (Wittebolle at al. 2009; Werner et al. 2011). The morphological diversity index could be performed by microscopy, including the Simpson's index (Hill 1973; Hunter and Gaston 1988). The microorganisms could be

classified by the digital software morphologically. From those data, a diversity index could be calculated using Simpson's diversity index (1-D) formula:

$$Diversity_i = 1 - D(Simpson's Diversisty Index) = 1 - \frac{\sum n(n-1)}{N(N-1)}$$

Equation 8 Simpson's biodiversity index

In equation 8, 'i' refers to methanogens (Meth) or total cells (Tot). N is the total number of organisms of all species and n is the total number of organisms of a particular species. The value of this index (unit-less) ranges between 0 and 1; the greater the value is, the greater the diversity of the microbial sample.

Methanogenic diversity index was calculated from 10 classes as shown in table 7. This morphological classification was based on literatures (Boone et al. 1993; Whitman et al. 2001) and own experiences as mentioned at table 6. These classes cannot be used for systematic assignment as shown in table 6. They can be used only for a rough estimation of morphological biodiversity.

Table 7 Tentative classification of the methanogenic diversity based on morphological properties

1	2	3	4	5	6	7	8	9	10
Coccoids	Coccoids	Coccoids	Coccoids	Rods	Rods	Rods	Rods	Filament	Packets
Length <0.5µm	Length >0.5µm and <1µm	Length >1µm and <2µm	Length >2µm and <10µm	Length <1µm	Length >1µm and <6µm Rods	Length >6µm and <15µm Rods	Length >15µm	Straight sheathed	Multi-cellular

The diversity index of non-fluorescent total cells was obtained from 3 classes as shown in table 8:

Table 8 Tentative classification of the morphological diversity of non-fluorescent total cells

1	2	3
Coccoids	Rods	Long Rods
Aspect < 1.5	Aspect 1.5< and <3	Aspect >3

Aspect means the ratio between the major and minor axes of the ellipse equivalent to an object: rods have a ratio higher than 1.5 and coccoids have a lower ratio than 1.5.

The morphological diversity of microorganisms in anaerobic digesters was tested in practice. For instance, temperature and substrate greatly influenced morphological diversity (figures 46 and 47).
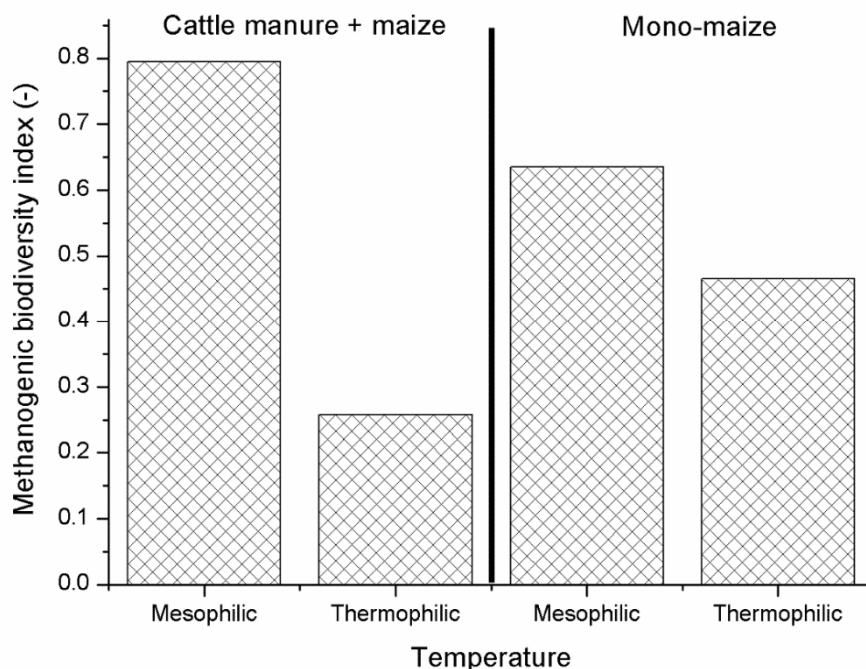
Figure 46 Example of morphological biodiversity index depending on temperature and substrate

Mesophilic digesters (37°C, Left: Seth storage tank with cattle manure + maize, Right: Lanken with mono-maize) showed a higher morphological microbial diversity than thermophilic digesters (53°C, Left: Seth F3 with cattle manure + maize, Right: Brekendorf with mono-maize). This result was interesting as it was in good accordance with molecular analysis data (Sekiguchi et al. 1998; Karakashev et al. 2005; Levén et al. 2007). The simultaneous addition of a co-substrate like cattle manure to the main substrate maize improved the biodiversity. However, this co-substrate negatively influenced the morphological diversity under thermophilic conditions in this case. It remains open, if these results would be generalised.
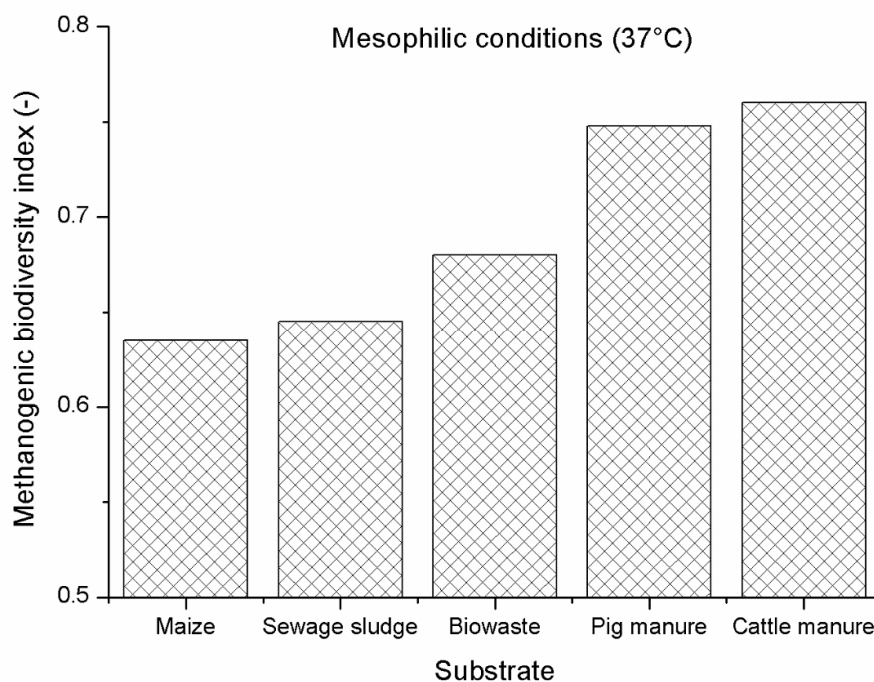
Figure 47 Example of morphological microbial diversity index depending on the used substrate in mesophilic anaerobic digesters

Animal manure (pig manure: Viersen, cattle manure: Seth) generated a higher morphological diversity. Biowaste (Bützberg) and sewage sludge (Geestacht) gave intermediate results, while mono-maize (Lanken) showed the lowest diversity. It was difficult to perform a comparison of molecular analysis on the archaeal community depending on different single substrate in anaerobic digesters, since biogas plants were normally fed by a mix of substrates (Nettmann et al. 2010). Cattle manure even as a mono-substrate could have different biodiversity, probably depending on the fodder of the cows (St-Pierre et al. 2013). A study compared microbial community in lab-scale reactors with different feedstock and concluded that the bacterial community was mainly influenced by the feedstock as well as the temperature, but the archaeal community was less correlated (Ziganshin et al. 2013). It was difficult to find a clear correlation of biodiversity between morphological analysis and molecular analysis due to the complexity of influencing factors and a restricted publications about it.

This biodiversity index could be involved as the strengthening factor on the inhibiting term of the VFA/(VS_Degradation_efficiency) exponentially. A higher biodiversity index was assumed to influence the TMCP more positively and the lower diversity negatively affected the TMCP. It should be remembered that a bioprocess with a low

diversity of a dominating functional group could also be stable (McCann 2000). It was e.g. observed in our laboratory that a maize-fed digester with a very low methanogenic diversity could show a stable process. In general, morphology of methanogens was much simpler than that of total cells. Therefore, the methanogenic biodiversity index was empirically applied, and softened with an extenuating factor (equation 9). Thereby a modification for the stabilising effect of VS (f_S) in relation to VFA was empirically done by multiplication with 100 to the VS (see figure 55, p97). However, biodiversity for the term total cells/methanogens was applied exponentially, without any extenuating factors. It could be mathematically formulated as in the equation below:

$$\frac{\left(1 - \frac{VFA}{VS * Degradation\_efficiency * f_S}^{1-e^{-2*Diversity\_Methanogens}}\right) * TMCP}{\frac{TotalCells}{Methanogens}^{Diversity\_TotalCells} + \left(1 - \frac{VFA}{VS * Degradation\_efficiency * f_S}^{1-e^{-2*Diversity\_Methanogens}}\right) * TMCP}$$

Equation 9 Involvement of the biodiversity index exponentially into equation 7

Terminating the equation of the indicator to describe the methanogenic activity

$$QMFI = \frac{\left(1 - \frac{VFA}{VS * Degradation\_efficiency * f_S}^{1-e^{-2*Diversity\_Methanogens}}\right) * TMCP}{T_T * \frac{TotalCells}{Methanogens}^{Diversity\_TotalCells} + \left(1 - \frac{VFA}{VS * Degradation\_efficiency * f_S}^{1-e^{-2*Diversity\_Methanogens}}\right) * TMCP}$$

Equation 10 Quantitative microscopic fingerprinting index as a result of equations 4 – 9

Now, only T_T was added in equation 9 in order to make the same unit as TMCP. T_T = equivalent TMCP for non-fluorescent total cells, its value is 1 [1/cm]. In conclusion, the QMFI equation [unit-less] was established in equation 10. The QMFI was made empirically based on several microscopic and chemical analysis data. Additionally, some fineness factors of biodiversity were supplemented.

Example calculation: Total cells $3.9*10^{10}$ (1/mL), methanogenic cell counts $2.1*10^9$ (1/mL), VFA (mg/L) = 877, VS = 8.9% ($\approx 8.9 *10^4$ mg/L, additionally multiplied by 100 for the stabilising factor of VS (f_S), Degradation efficiency = 0.8 (or 80%), diversity of total cells = 0.59 (or 59%), diversity of methanogens =0.53 (or 53%), Total cells/methanogens = 18 (-), TMCP = 41 (1/cm) from $2.1*10^9$ (methanogens, $1/cm^3$) * 0.84 (fluo-intensity) * 2.36 (cell area, μm^2) /10^8.

Consequently, QMFI = $(1-(877/(8.9*10^4*100*0.8))^{\wedge}(1-EXP(-2*0.53)))*41/(18^{\wedge}0.59+(1-(877/(8.9*10^4*100*0.8))^{\wedge}(1-EXP(-2*0.53)))*41) = 0.88$.

In summary, the QMFI is made of three parts: methanogenic activity, chemical habitat data (environment) and biodiversity. The methanogenic activity was represented with mainly the methanogenic cell counts including their size and fluorescence intensity. The chemical habitat data mean chemical components as substrate or products of cellular activity. The biodiversity indicates capability of adoption of microbes to the environment. From the complex interaction between methanogens, chemical habitat data and biodiversity, the methanogenic indicator was created.
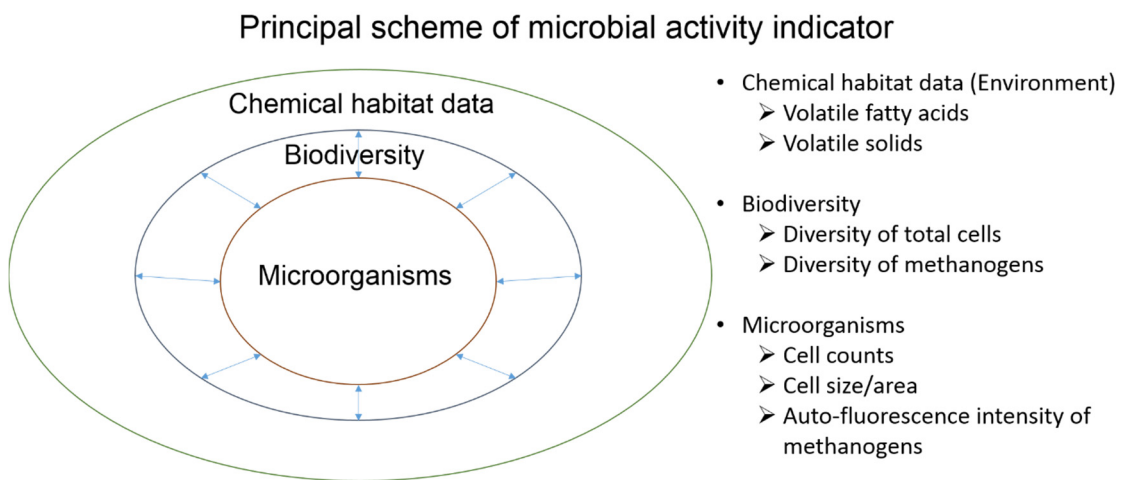
Figure 48 Principal scheme of the microbial activity indicator developed and used for establishment of QMFI

To our knowledge, it is the first approach to combine cell counts with chemical habitat data including VFA and biodiversity in order to find and objectify the methanogenic indicator for the performance of anaerobic digesters. Further investigation and optimisation of the indicator is requested in the future.

3.5. Validation of the QMFI

Assessment of microbial quality in biogas process is a complex process and can be expressed in various ways. In this study, a microscopic method was developed which was called the quantitative microscopic fingerprinting index (QMFI). The QMFI is an empirical value and varies between 0 and 1, meaning that a higher QMFI resulted in higher methanogenic quality. The QMFI is calculated by a mathematical equation consisting of 10 parameters, including total cell counts (N/mL), methanogenic cell counts (N/mL), ratio of total cells to the methanogens (%), methanogenic fluorescence intensity (%), mean value of methanogenic single cell area (μm^2), volatile solid content of fermentor medium (VS, mg/L), volatile fatty acid of fermentor medium (VFA, mg/L), degradation efficiency (%), biodiversity of total cells (0-1) and biodiversity of methanogens (0-1) as shown in equation 10. All of the parameters except VS, degradation efficiency and VFA can be measured by a fluorescence microscope including digital image analysis software. The main part of the equation is a product of methanogenic cell number (N/mL) x methanogenic fluorescence intensity (%) x mean value of methanogenic single cell area (μm^2), describing the total methanogenic cell power (TMCP). This TMCP is regulated (retarded) by a term of the ratio of total cells to the methanogens which indicates a higher tendency to accumulation of VFA by increase of non-fluorescent cells (higher activity of hydrolysis and fermentation step). The TMCP is simultaneously influenced by metabolic factors like VFA (mg/L), VS (mg/L), and degradation efficiency (%), in order to describe a more valid relationship between VFA and cell counts. A higher VFA content resulted in a decline in TMCP. However, this term for metabolic state is strengthened exponentially by the biodiversity index (0-1, where 1 indicates the highest diversity) since higher biodiversity is assumed to support a better stability of fermentation and the capability of adaptation to the inhibiting conditions.

The QMFI was tested in practice. Firstly, the QMFI was analysed in an agricultural large-scale biogas plant in Germany in order to see changes of QMFI caused by some accidental occurrences like the accumulation of propionic acid over time. In addition, the relationship between specific gas production rate and QMFI was investigated in a similar large-scale biogas plant, as well as in a lab-scale biogas reactor fed by fodder beet at different organic loading rate. Finally, the QMFI was measured in serially connected large-scale digesters, with a concomitant decrease of VS, in order to determine the relationship between QMFI and VS.
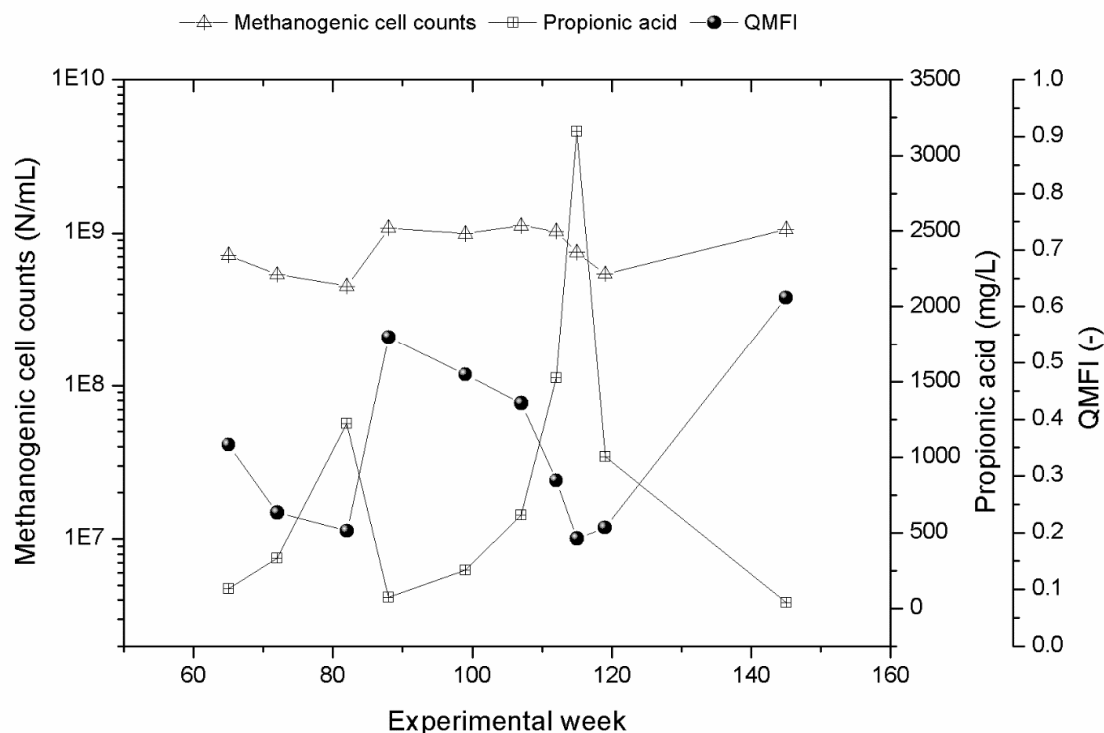
Figure 49 Variation of the quantitative microscopic fingerprinting index (QMFI) and methanogenic cell counts with respect to accumulation of propionic acid

Propionic acid concentration increased at experimental week 82, from 128 to 1221 mg/L and at experimental week 115, dramatically from 73 to 3160 mg/L due to overloading of substrate (grass). Methanogenic cell counts varied between 7.2E+8 and 1.1E+9. Methanogenic cell counts reacted to the inhibiting propionic concentration. However, its change was relatively small. At the highest concentration of propionic acid, methanogenic cell counts were reduced from 1.03E+9 to 7.51E+8, which was a reduction of about 27%. Of importance was the relative stable methanogenic cell counts and the obvious change of QMFI between experimental week 88 and 112. In this period, propionic acid concentration began increasing, consequently QMFI started decreasing while methanogenic cell counts did not change much. Methanogens reacted to the accumulation of propionic acids relatively lowly. The QMFI oppositely reacted to the propionic concentration obviously. At the highest concentration of propionic acid, the QMFI was reduced from 0.55 to 0.19, which was a reduction of about 65%, representing a higher sensitivity compared to methanogenic cell counts (27%).

The next diagram shows a relationship between the QMFI and volatile fatty acid concentration as well as between methanogenic cell counts and the specific biogas production rate in fermentor F2 of the full-scale biogas plant Seth.
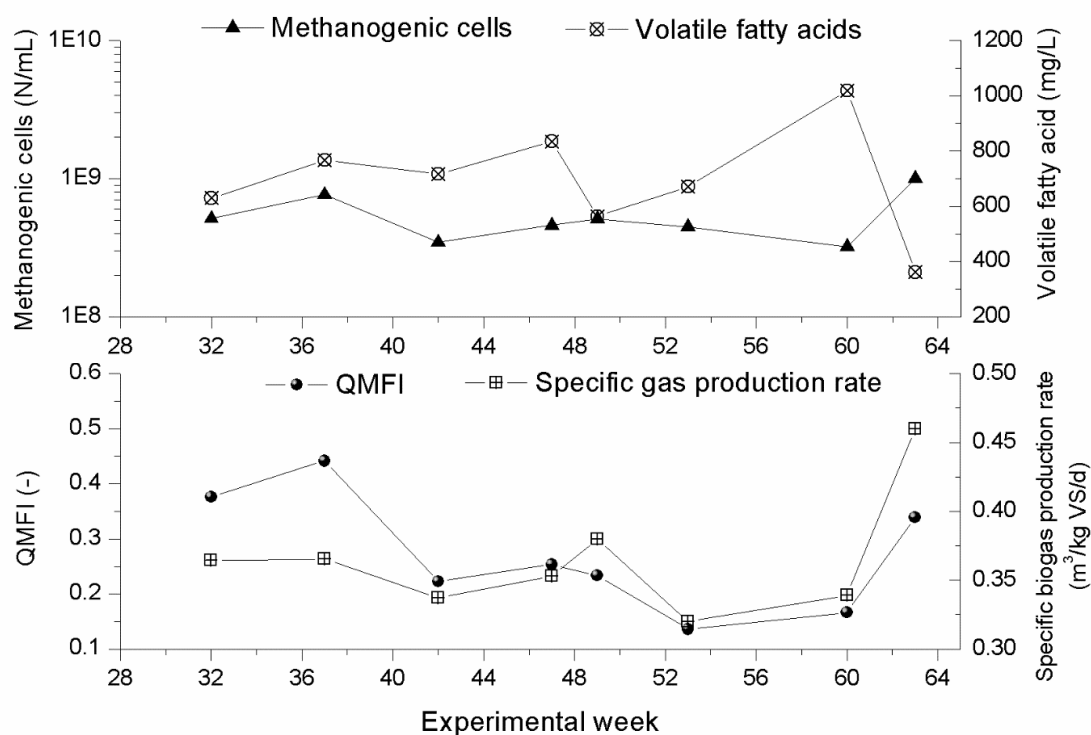
Figure 50 Relationship between QMFI and methanogenic cell counts, volatile fatty acid, and specific biogas production rate in fermentor F2 of the biogas plant Seth

Specific biogas production rate was measured in the large-scale agricultural biogas plant Seth during 31 weeks operation and compared with QMFI. Specific biogas (CH_4+CO_2) production rate varied between 0.32 and 0.46 ($m^3/kgVS*d$). Generally, volatile fatty acid concentration were relatively low below 1100 mg/L, showing no significance influence. The curve of QMFI showed a similar tendency as the curve of specific biogas production rate. The highest value of specific biogas production rate was found in experimental week 63, where the highest methanogenic cell number was found at 1.01E+9. But in this week, methanogenic auto-fluorescence intensity was extremely low at 19%. If the fluorescence intensity would be 40%, the QMFI would be 0.52, meaning the highest value would happen. The reason of the lower intensity in the last three weeks (53, 60, and 63) was unclear. Similarity of the curves between methanogenic cell counts and specific biogas production rate was higher (Pearson's correlation coefficient 0.87, Pearson, 1920) than curves between QMFI and specific biogas production rate (Pearson's correlation coefficient 0.51). Methanogenic activity being described by QMFI was positively proportional to the specific biogas production rate but it seemed to be not necessarily as same as methanogenic activity represented by specific biogas production rate.

Another comparison between the QMFI and specific biogas production rate was performed by analysing a laboratory reactor fed with only fodder beet in figure 51. The reactor was operated at 58°C by Sebastian Antonczyk of the laboratory. Organic loading rate was increased stepwise from 5 to 13 kg VS/m^3/d. Other detailed measurement data can be found in the appendix.
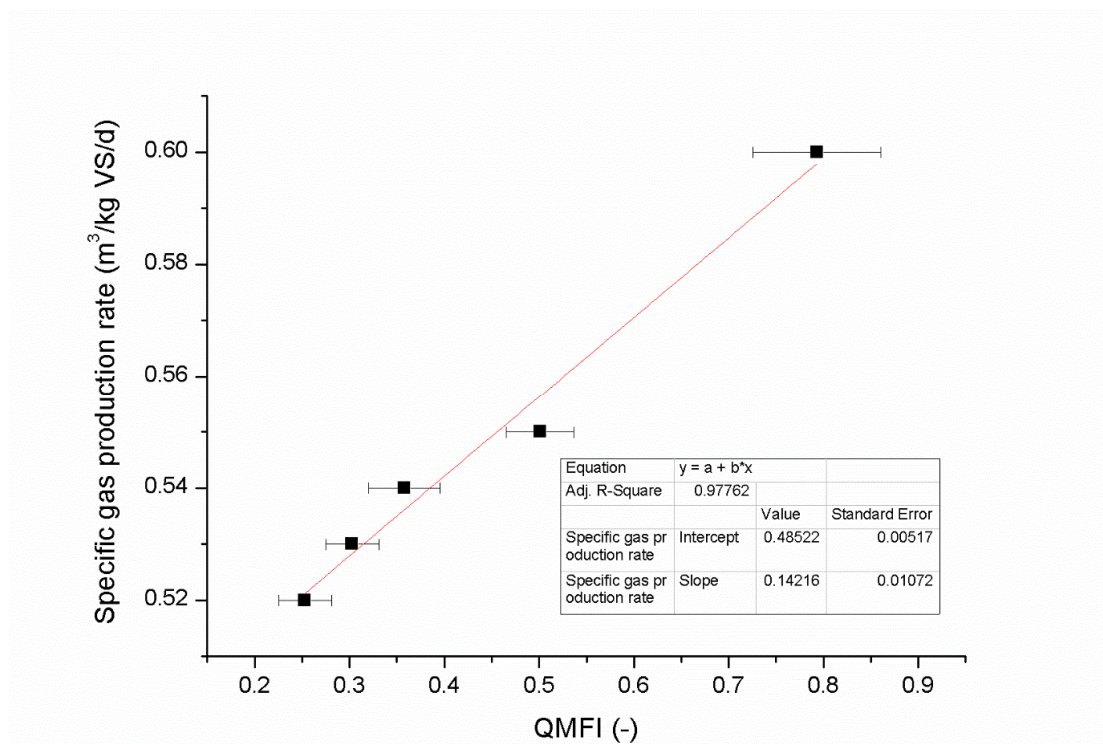
Equation	y = a + b*x		
Adj. R-Square	0.97762		
		Value	Standard Error
Specific gas production rate	Intercept	0.48522	0.00517
Specific gas production rate	Slope	0.14216	0.01072

Figure 51 Relationship between specific gas production rate and quantitative microscopic fingerprinting index (QMFI) for a laboratory reactor (RM60) fed with fodder beet

An increase of organic loading rate from 5 to 13 (5, 7, 9, 11, 13) kgVS/m3/d caused a decrease in hydraulic retention time of 14.6, 11.8, 8.4, 8.3, and 8.7 days, respectively. Organic loading rate was stepwise increased after obtaining a stable run of about 1-2 weeks. The fluorescence intensity was relatively low at 28%. It could be seen that mono-rod-type methanogens were dominantly found during all periods. A very good correlation between specific biogas production rate and the QMFI was found with R2= 0.977, meaning that the QMFI was a proper indicator of the biogas production rate in this case. Interestingly, nearly the same correlation coefficient (R^2) could be found between methanogenic cell counts and specific biogas production rate as well. The horizontal error bars indicate standard deviation of QMFI being 7-11% (N=20 pictures per sample).

Figure 52 shows a dependence of cell counts and QMFI on the volatile solid content of fermentors. The investigated biogas plant Lanken has four serially-connected digesters.

Maize as a mono-substrate was fed to the digester and digested stepwise further on in subsequent digesters as post-digester 1, post-digester 2 and digestate storage.
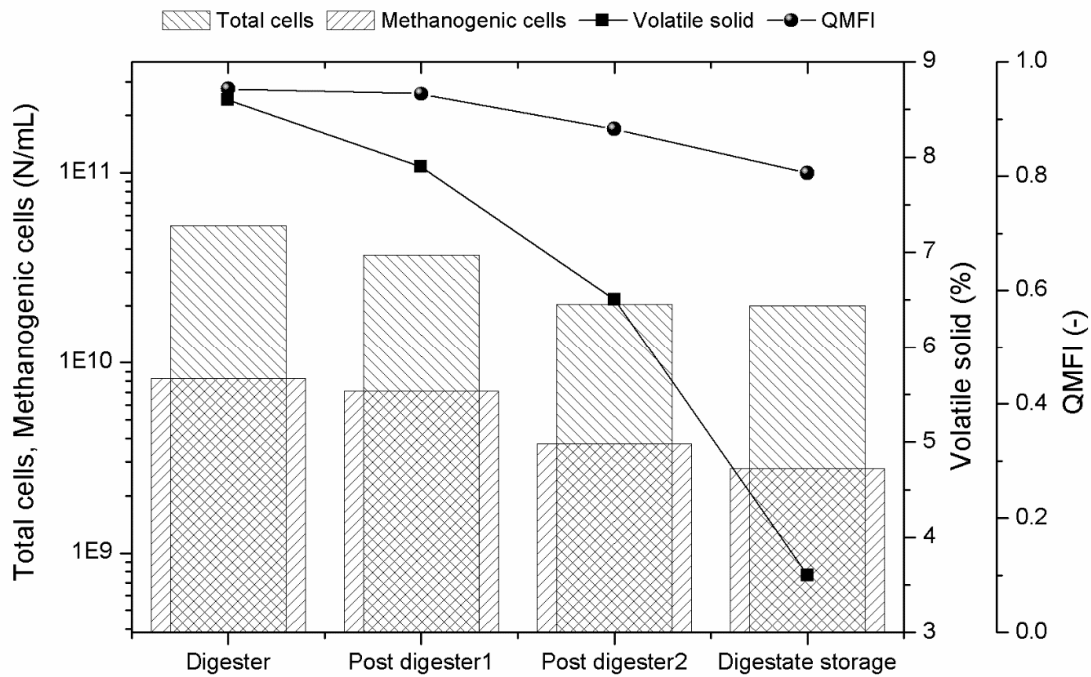
Figure 52 Change of total cells, methanogenic cell counts, and QMFI depending on decrease of the volatile solid content in serial connected digesters of a full-scale biogas plant Lanken

Volatile solid content was stepwise reduced from 8.6, 7.9, 6.5, and 3.6% respectively. Along with this decrease of volatile solids, total cell counts as well as methanogenic cell counts decreased. In other words, the highest activity of methanogens was expected in the first digester, where the highest volatile solid content was present. The QMFI was also stepwise reduced in the same way as volatile solids content and methanogenic cell counts. Other chemical data can be found in the appendix.
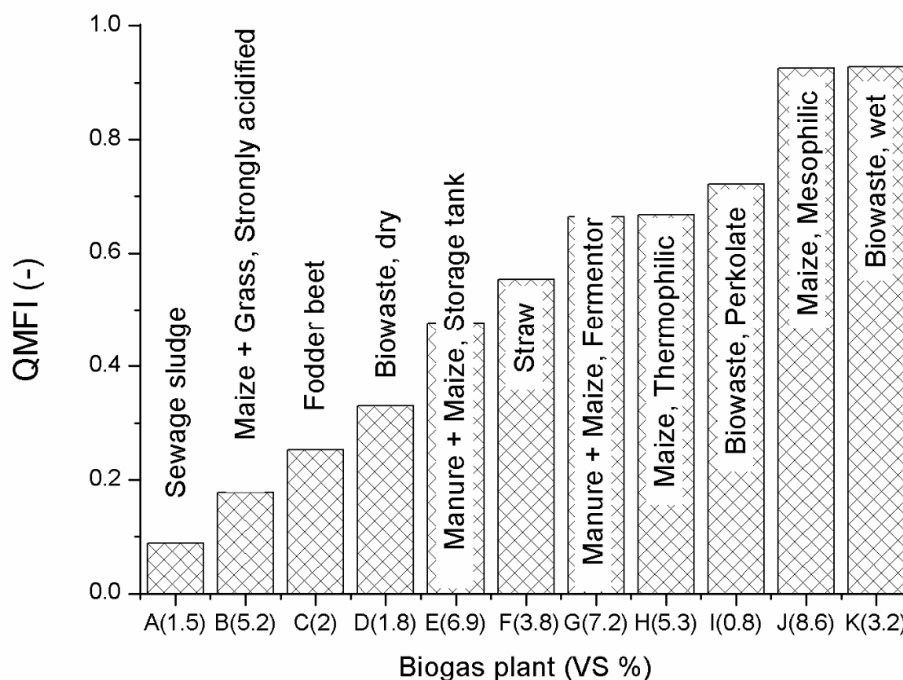
Figure 53 Quantitative microscopic fingerprinting index depending on operational conditions. (Number) indicates volatile solids content of fermentor (VS %). Detailed data of 11 fermentors in appendix (7.3.)

In figure 53, QMFI was determined and arranged with respect to operational conditions including substrate, temperature, and digestion step. Sewage sludge (A, Geestacht) showed the lowest value of QMFI (0.09) while a municipal biowaste plant (L, Biowerk) at 37°C exhibited the highest QMFI value (0.928), similarly in maize fed plant in mesophilic condition (K, Lanken). Interestingly, digester B (Parkstetten) with maize and grass feeding should probably have a QMFI of 0.45 when showing a stable state with VFA concentration of e.g. 1000 mg/L. However, digester B (Parkstetten) had a very high VFA content of 22900 mg/L at that time, i.e. the process was highly acidified in reality (mainly propionic acids). The QMFI of digester B (Parkstetten) was 0.131, strongly declined by this high content of VFA. The QMFI of biowaste in dry fermentor (D, Bützberg) was 0.347. The same substrate cattle manure + maize but in different digestion step as storage tank after main digestion (E, Seth) and main digester (G, Seth) showed different QMFI values of 0.477 and 0.664, respectively. Based on figure 53, a ranking table for QMFI was established, table 9.

Table 9 Tentative order of quantitative microscopic fingerprinting index (QMFI) to indicate a good or bad performance of a biogas reactor

Scale of QMFI	Assessment
QMFI >0.85	Very Good
QMFI >0.7 and < 0.85	Good
QMFI >0.5 and <0.7	Satisfying
QMFI >0.3 and <0.5	Acceptable
QMFI >0.15 and <0.3	Bad
QMFI <0.15	Very bad

According to our experience, the scale of the methanogenic quality was tentatively made according to the QMFI in table 9. When the QMFI was higher than 0.85, e.g. mono-maize or maize + triticale or biowaste (wet fermentation) in mesophilic conditions, the methanogenic quality should be very good, including a high number of active methanogens of above 3E+9. Fermentors containing few methanogens at a low volatile solid content showed the lowest QMFI value of below 0.1, indicating a very bad state of methanogenic quality or a very low potential for biogas production. The advantage of the QMFI was the sensitivity of its values. The QMFI values were very sensitive according to various conditions down to three decimal points, and simultaneously widely occurred in the whole area between 0 and 1. The QMFI was a sensitive indicator based on cell counts, including other chemical and microscopic data; however, it had some limitations. Some phenomena could not be explained by all of the chemical data as well as by the QMFI. However, the QMFI is the first attempt to combine microscopic data with chemical data to obtain an improved monitoring method of anaerobic digesters. The QMFI could hopefully be used for a rapid monitoring method in cooperation with existing chemical and molecular analyses.

3.6.   Simulation of the QMFI

The impact of main components of QMFI including volatile solid content, volatile fatty acid and methanogenic cell counts on the QMFI was simulated. 11 real biogas plants were chosen and selected (arranged) by their different volatile solid contents which are one of the essential influencing factors (appendix 7.3.). Authentic biogas plants were chosen as reference to represent a realistic effect of complex interaction of the 10 parameters of the QMFI. All other parameters were taken from the real biogas plants except volatile fatty acid or methanogenic cell counts which was varied artificially for simulation to see its effect on the QMFI value (figure 54, 55, 56).
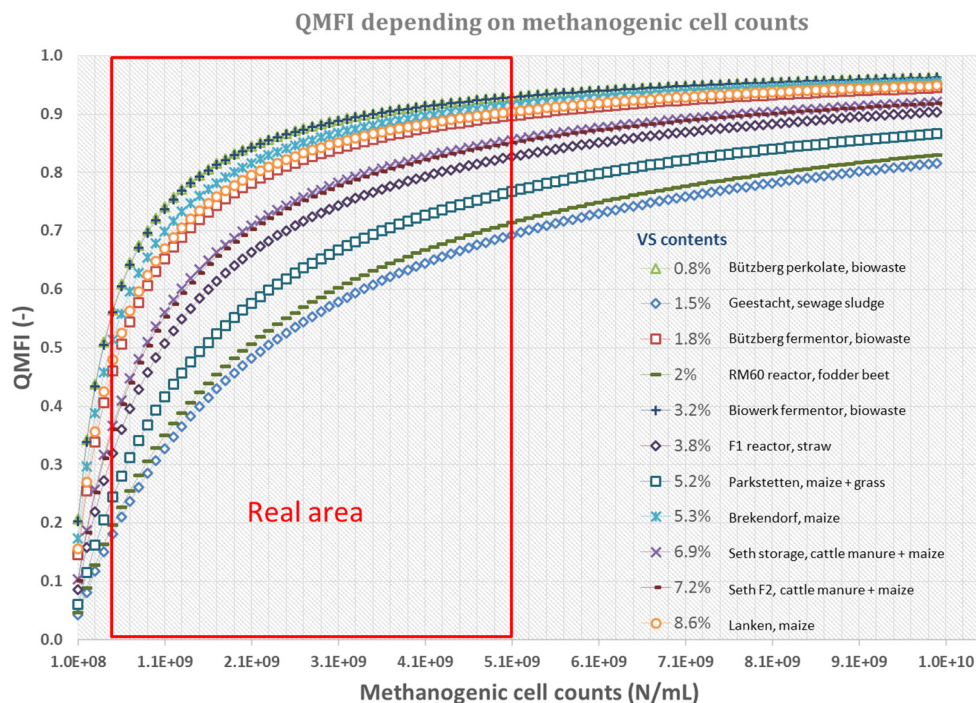
Figure 54 Simulation of the effect of varied methanogenic cell counts on the QMFI. 11 different real biogas plants were chosen as reference, containing different volatile solid contents (VS). Detailed data of 11 biogas plants, appendix 7.3.

The QMFI was simulated depending on methanogenic cell counts (figure 54) varied artificially from 1E+8 to 1E+10. All other parameters for calculation of QMFI were taken from 11 real biogas plants, except the methanogenic cell counts. 11 biogas plants were chosen and arranged after volatile solid content (VS). Generally, methanogenic cell counts found in real biogas plants varied approximately between 3E+8/mL as minimum and 5E+9/mL as maximum (real area in red square) so far, most generally between 5E+8 and 3E+9 according to own analysis on more 200 biogas fermentors (approximately

107100 pictures, 150 gigabyte). Influence of methanogenic cell counts on QMFI was very high, showing an exponentially steep curve in this range from 5E+8 to 3E+9. The slope of curves were very differently, not necessarily dependent on the VS. The most sensitive and steep curve was found for the biowaste treatment plants Bützberg (solid state fermentation) and Biowerk (liquid state fermentation). Maize-fed fermentors like Brekendorf and Lanken were secondly influenced by the methanogenic cell counts. The most flat curve was shown for the waste water treatment plant Geestacht with its sewage sludge.
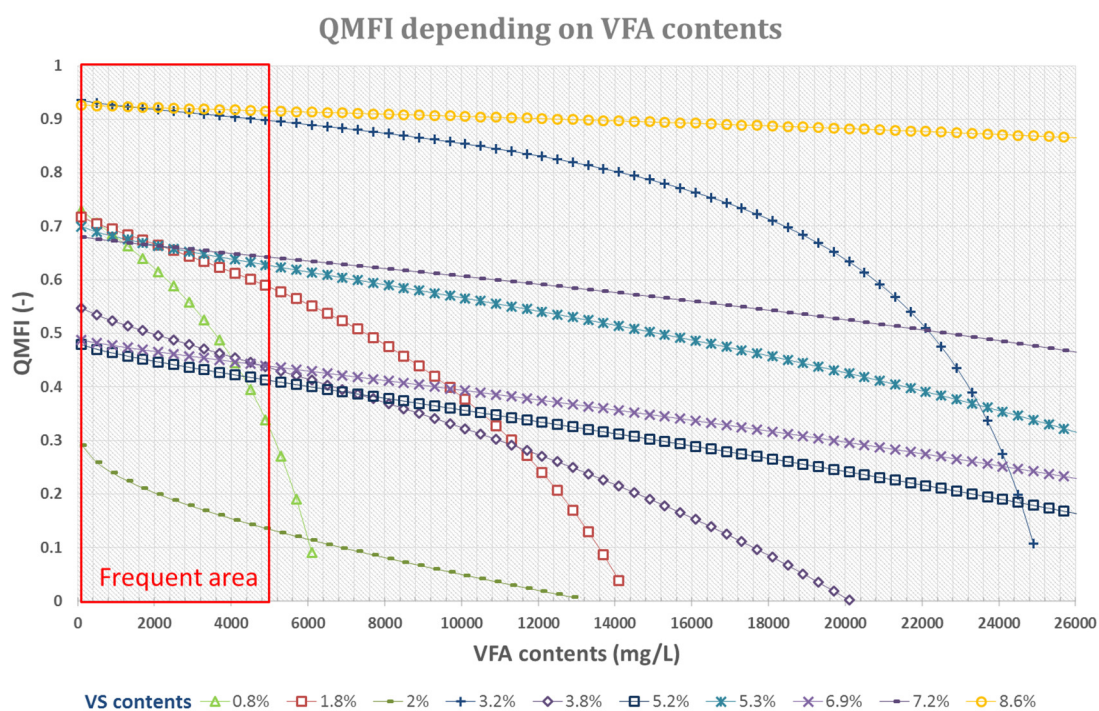
Figure 55 Simulation of the effect of varied volatile fatty acids (VFA) on the QMFI. 11 different real biogas plants were chosen as reference, containing different volatile solid contents (VS). Detailed data of 10 biogas plants, see appendix 7.3.

QMFI was also simulated depending on varied volatile fatty acid concentration between 0 and 25000 mg/L. VFA was found normally in stable running biogas plants, e.g. below 1000 or 2000 mg/L. However VFA can increase due to e.g. overloading or inhibition of bioprocess up to 25000 mg/L in plant B Parkstetten (appendix 7.3.). Normally, VFA was found in a range between 100 and 5000 mg/L (frequent area in red square). More than 2000 mg/L of VFA can indicate an acidified fermentor, in which the bioprocess can nevertheless show a stable run depending on the buffer capacity. Generally, VFA concentration influenced QMFI negatively but in different ways (different slopes of curves). At fermentors containing higher VS contents more than 5%, the impact of VFA

was not so significant, representing a flat slope of curves. The influence was diminished, probably due to sufficient stabilizing biomass as well as buffer capacity. That can mean higher probability to degrade VFA quickly in order to stabilize the system again. At fermentors with lower VS contents, QMFI was strongly dependent on VFA, showing a sharp decline of QMFI.

Additionally, the QMFI was simulated by changing 9 parameters to see their influence.
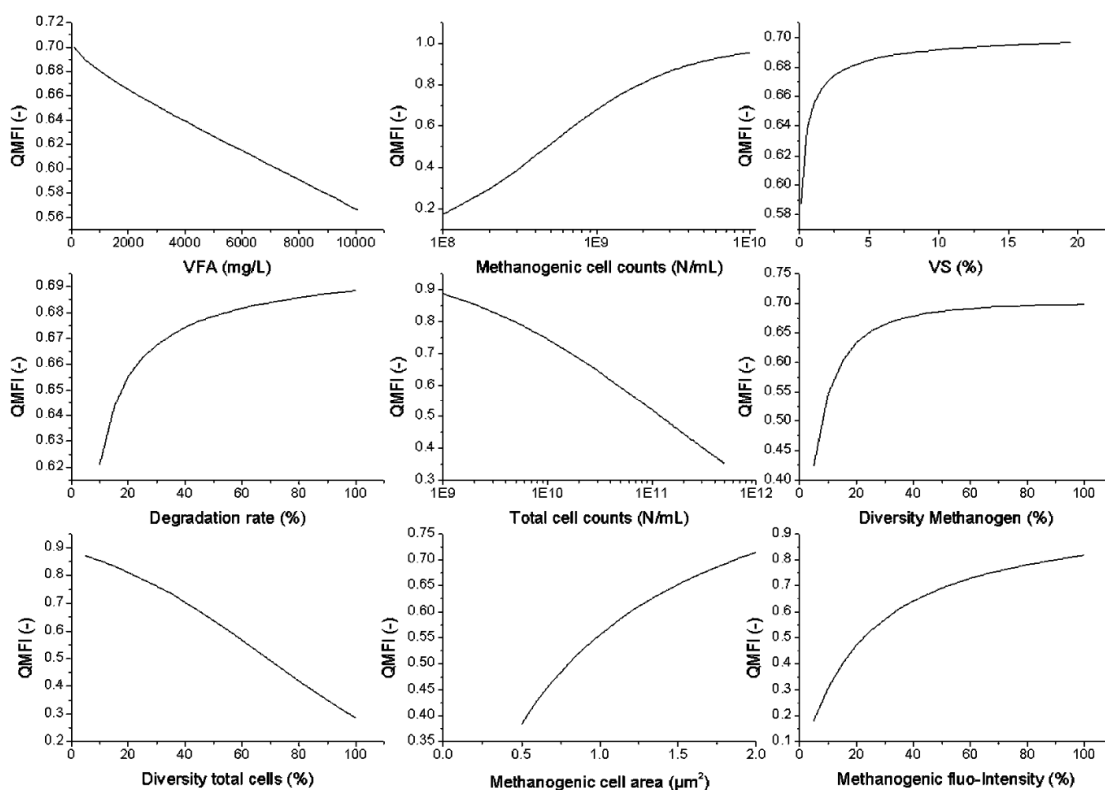
Figure 56 Simulation of the QMFI depending on the parameter: 9 parameters were varied at a biogas plant sample fed with maize under thermophilic condition (53°C), volatile solid content of digester was 5.3%, Brekendorf (see appendix 7.3)

The impact of each parameter on the QMFI was shown by simulation in figure 56. A biogas plant (Brekendorf) fed with maize was chosen for simulation as a reference, which approximately showed average values of all parameters. The details of measured parameters are listed in a table of appendix 7.3. Three parameters like VFA (volatile fatty acids), total cell counts and diversity of total cells influenced the QMFI negatively while other parameters of methanogenic cell counts, volatile solid contents (VS), degradation efficiency, diversity of methanogens, methanogenic cell area and methanogenic fluorescence intensity showed a positive effect with different strength. Methanogenic cell counts and their intensity influenced the QMFI mostly among the positive influencing parameters while VS and degradation efficiency showed a slight influence. Total cell

counts and their diversity influenced stronger than VFA contents among the negatively acting parameters.

Chapter 4: Results and Discussion for Simplified Isolation Techniques for Methanogens

No great discovery was ever made without a bold guess

Isaac Newton

4. Results and discussion of the optimised cultivation of methanogens

4.1. Optimisation of cultivation and isolation technique

First of all, the ingredients for an optimal cultivation medium were searched. Five different DSM medium recipes (141, 287, 318, 332, and 334) and four different reference species (DSM 1498 *Methanoculleus marisnigri*, DSM 3915 *Methanoculleus thermophilus*, DSM 3267 *Methanothermobacter thermoautotrophicus*, and DSM 2133 *Methanothermobacter marburgensis*) were used for this purpose. Finally, we modified and optimised existing recipes and created a HAW-medium, in which rod-type methanogens as well as coccoid-type methanogens could grow under thermophilic (53°C) and mesophilic (37°C) conditions, as well as at 20°C and 26°C to find different types of methanogens depending on the temperature. In this study, rod-type methanogens grew faster than coccoid-type methanogens, i.e. it was easier to cultivate rod-type methanogens. Fourteen different sources (inoculum, table 2) were inoculated into the HAW-medium in duplicate or triplicate and further transferred. To enrich target methanogens after checking by microscopy, dilution series for each inoculum were performed using different media.

In the initial phase, many failures occurred when working anaerobically, i.e. the exclusion of oxygen and gas tightness at each step were not perfect. All media were stored with a slight overpressure of N_2 (0.25 - 0.5 bar) to avoid introduction of air into the bottle with medium. Another problem was precipitations in the medium. Thus the amount of phosphate and carbonate had to be optimised (reduced) repeatedly because phosphorous and carbon dioxide are essential nutrients but they caused precipitations. In terms of precipitation, an alternative pH buffer was chosen, imidazole, which unfortunately also caused precipitation. Therefore, the amount of imidazole was reduced upon 10 mM. Afterwards, a clear medium was obtained. In the preparation of medium, pH control was also an important issue to consider during cultivation. To enrich target methanogens, two different pH values were used: 6.4 and 7. Sodium sulphide was generally used as a reducing agent and S-source, but its pH value is very high in solution at 9. Therefore, after adding sodium sulphide, the pH change should be taken into account. Another important point was the redox potential, which should be kept below –350 mV. Cysteine, sodium sulphide and titan citrate were generally used as the reducing agents in this study. Titan citrate was the strongest reducing agent while cysteine was the weakest. Especially for reducing solid medium, sodium sulphide was the optimal reducing agent because

sodium sulphide could be easily changed to hydrogen sulphide gas, which can move into the entire space of the anaerobic cultivation box. But it was difficult to obtain the right concentration of H_2S (check by nose). An advantage of cysteine was its low toxicity, while high amounts of sodium sulphide and titan citrate could be toxic (Wachenheim and Hespell 1984; Hilton and Oleszkiewicz, 1988). Their toxicity was not tested in this study. Regarding sodium sulphide, several points should be noted. Sodium sulphide is very easily oxidised with air and is volatile. It should be saved in a gas-tight bottle and made freshly every month. The solubility of sodium sulphide depends on pH and temperature. At a lower pH and higher temperature (e.g. during thermophilic cultivation), the solubility of sodium sulphide is decreased and the S-source will be reduced for methanogens in liquid medium. Also, the toxicity of hydrogen sulphide can increase (Pender et al. 2004). Occasionally, it was necessary to boost the growth of methanogens by organic compounds. For this purpose, yeast extract or trypticase could be added (Ferguson and Mah 1983), but it should be carefully considered that other bacteria were also stimulated. Thus, yeast extract and trypticase were applied only in monoculture or in the case of solid medium. There was another option for stimulating the growth, i.e. sludge fluid or fermentor medium (Stantscheff et al. 2014), but sterilisation of the fermentor medium was not easy due to condensed soil particles. After a long period of centrifugation for four hours and using 2-3 repeated filtration steps, a relatively clear fermentor medium could be achieved. Another problem was sterilisation of the medium. Some components were heat unstable, like vitamins and cysteine. After autoclaving, the precipitation of phosphate or carbonate with e.g. trace elements often happened, which were then biologically not available. Thus, membrane filtration with a vacuum pump was used as the main method for sterilisation.

Optimisation of medium constituents

In table 10, the HAW-medium was compared with five other media (DSM 141, 119, 287, 318, and 332) for the cultivation of methanogens.

Table 10 Comparison of six media recipes for cultivation of methanogens

Macro-elements	HAW	DSM 141	DSM 119	DSM 287	DSM 318	DSM 332
	g/L	g/L	g/L	g/L	g/L	g/L
$(NaPO_3)_x$	0.025					
KH_2PO_4			0.5	0.3	0.3	
KCl	4.8	0.34				
NH_4Cl	1	0.25	0.4	2.7	1	1
$(NH_4)_2SO_4$				0.3		
$K_2HPO_4*3H_2O$		0.2		0.3		0.4
NaCl	1.75	18	0.4	0.61	0.6	
$MgCl_2*6H_2O$	1	3			0.1	0.1
$MgSO_4*7H_2O$		3.45	0.4	0.13		
$CaCl_2*2H_2O$	0.073	0.14	0.05	0.14	0.08	
$FeSO_4*7H_2O$			0.002			
$Fe(NH_4)_2(SO_4)_2*7H_2O$		0.002				
Fe(II)Citrate	0.0024					
Sodium formate	0.68		2			5
Sodium acetate	0.41	1	1	2.5		1
Yeast extract	5[a]	2	1		0.5	1
Trypticase	5[a]	2			0.5	1
Resazurin	0.002	0.001	0.001	0.001	0.001	0.001
Vitamin solution	10 mL	10 mL[b]		10 mL[b]	10 mL[b]	10 mL[b]
Fatty acid solution			20 mL			
$NaHCO_3$	0.42	5	4	5		
$KHCO_3$					2	
Methanol					5 mL	
Na_2CO_3						1.5
Na_2S*9H_2O	0.24	0.5	0.5	0.3	0.3	0.2
Titan citrate	38 mg Ti, 4 mg Citrate					
Imidazole	0.68					
Trace elements	10 mL	10 mL[b]	1 mL[c]	10 mL[b]	10 mL[d]	10 mL[b]
Cysteine-$HCl*H_2O$	0.15	0.5	0.5	0.3	0.3	0.5
Sludge fluid			50 mL			
pH adjusting	1M HCl	10M KOH		H_2SO_4		
Gas phase	H_2/CO_2, **2 bar**	H_2/CO_2, 2 bar	H_2/CO_2	H_2/CO_2, 1.2 bar	H_2/CO_2, 1.2 bar	H_2/CO_2, 2 bar

a: only for monoculture and solid medium

b: DSM Medium 141

c: DSM Medium 320

d: DSM 318

A special property of the HAW-medium was the relatively high concentration (4.8g) of KCl, meaning 2.5 g of potassium (64 mM). According to Scherer (1983), elemental composition of potassium in 10 methanogens lay between 0.13% and 5% (1.3–50 g/L) representing 33 mM to 1.28 M potassium (Scherer et al. 1983). Generally, biogas plants fed by manure and energy crops contained a high amount of potassium e.g. 3 g/L found by own analysis. A study found a close correlation between the degree of syntrophic acetate oxidation with subsequent methanogenesis and the concentration of ammonium and potassium (Schnürer et al. 1999). Additionally, the oxidation of acetate to hydrogen and the subsequent conversion of hydrogen and carbon dioxide to methane was suggested as a dominant methanogenic pathway (Karakashev et al. 2006). On the other hand, too high concentrations of potassium could also inhibit anaerobic digestion (3–6 g/L, 77–154 mM), according to Chen and Cheng (2007). Such background was the reason for the relatively high concentration of potassium in the medium. Instead of a potassium based phosphate, polyphosphate was used as new phosphate source for methanogens in the HAW-medium. Because of precipitation, $NaHCO_3$ concentration was reduced on 5 mM in the medium. Additionally, reducing agent titan citrate was used.

Yeast extract was necessary for the cultivation of some methylotrophic methanogens (e.g. *Methanosarcina barkeri*) which was stored as a powder in a glass tube for a long-term, in our case for 32 years. Several attempts failed, but after the addition of yeast extract to the HAW-medium, the 32 years old cells were able to grow again.

Sulphate was intentionally avoided in the HAW-medium because of the possible enrichment of sulphate-reducing bacteria (Lovely and Klug 1983). The phosphate buffer was avoided due to potential precipitations. Instead of the phosphate buffer an Imidazole-buffer was used.

Table 11 Comparison of 4 recipes for trace elements

	HAW	DSM 141	DSM 318	DSM 320
Micro-elements	mg/L 1:100 Application	mg/L 1:100 Application	mg/L 1:100 Application	mg/L 1:100 Application
HCl (25%; 7.7M)				10 mL
$(NaPO_3)_x$	10			
Nitrilotriacetic acid		1500	12800	
$MgSO_4*7H_2O$		3000		
$MnSO_4*H_2O$		500		
NaCl		1000	1000	
$FeCl_2*4H_2O$				1500
$FeCl_3*6H_2O$			1350	
$Fe(NH_4)_2(SO_4)_2$				
$FeSO_4*7H_2O$		100		
$CaCl_2*2H_2O$		100	100	
$ZnSO_4*7H_2O$		180		
$ZnCl_2$	6.81		100	70
$MnCl_2*4H_2O$	1.98		100	100
H_3BO_3	0.62	10	10	6
$CuSO_4*5H_2O$		10		
$CuCl_2*2H_2O$	1.7		25	2
$NaWO_4*2H_2O$	3.3	10		
$KAl(SO_4)_2*12H_2O$		20		
$CoSO_4*7H_2O$		180		
$CoCl_2*6H_2O$	11.89		24	190
$NiCl_2*6H_2O$	11.88	30	120	24
$Na_2SeO_3*5H_2O$	2.63	0.3	26	
$Na_2MoO_4*2H_2O$	2.41		24	36

Also, a mild chelating agent, polyphosphate was used in the HAW-medium instead of nitrilotriacetic acid. The amounts of Zn, Mn, and Boric acid as well as Co, Ni, W, and Mo were reduced according to the experience of the lab with trace element supplementation. They were relatively low in the HAW-medium compared to other trace element recipes, as it was found that the regular concentrations of several μM could diminish the free available sulphide being sulphur source. The free sulphide concentration is often measured amperometrically, below 10 ppm (Scherer, unpublished).

Optimal Sulphur-Source

The sulphur source (S-source) for methanogens has been intensively investigated over the last few decades (Khan and Trottier 1978; Scherer and Sahm 1981; Mazumder et al. 1986; Scherer 1987; Zhang and Maekawa 1996; Liu et al. 2012) and is summarised in figure 57. S-source is essentially needed for many proteins e.g. methyl coenzyme M reductase which catalyses the last step of methanogenesis in combination with thiole coenzyme B: $Me\text{-}S\text{-}CoM + CoB\text{-}SH \rightarrow CH_4 + CoB\text{-}S\text{-}S\text{-}CoM$, $\Delta G^{\circ\prime} = -30$ kJ mol^{-1} (Deppenmeier et al. 1996). A favourite S-source was sodium sulphide because it could also be used as a simultaneous reducing agent. The optimal concentration of sulphide was thus investigated, which was dependent on pH, pressure and temperature. At neutral pH, about one third of the sulphide occurs as hydrogen sulphide in the gaseous form (Liu et al. 2012). If the cultures were performed under thermophilic conditions, the solubility of hydrogen sulphide was decreased e.g. solubility was about 2.7 times less at 60°C than at 20°C (3.8 g H_2S/kg H_2O). On the other hand, hydrogen sulphide is toxic beyond a certain concentration (McCartney and Oleszkiewicz 1991) more than 2 mM. Thus, the addition of a proper amount of sulphide was very important.

S-Source for Methanogens

All known methanogens can use sulphide (S^{2-}) as the sole sulphur source and many methangens also use S^0 as a sulphur source (produce high levels of sulphide from S^0 in the presence of H_2).
Microbial dissimilatory sulphate to sulphide reduction is the most common and widespread process for sulphur assimilation. But only *M. thermolithotrophicus* and *M. ruminatium* are able to use sulphate as a sole sulphur source.

Cysteine
Not toxic

Sulphate
Not toxic

Glutathione
Not toxic

Thiosulphate

Sulphite

Sulphur

Methionine, Thioacetic acid, FeS, Dithionite, Tetrathionate

Methanogens

S^{2-} ↔ SH^- ↔ H_2S toxic

Sulphur containing compounds from S^{2-}: Amino acids: Cys, Met, Vitamins such as Fe-S clusters, thiamine, molybdopterin, CoA, biotin, and coenyzmes.

$S^{2-} + 2H^+ ↔ SH^- + H^+ ↔ H_2S$
  Basic   Acidic
At neutral pH, 1/3 of sulphide occurs as H_2S.

Na_2S is generally used as a reducing agent. Sulphate, cysteine and glutathionine were applied and had positve effects on methanogenic growth. But CH_4 production and cell density were not convincing up to now.

Figure 57 Various sulphur source for methanogens

Cysteine, sulphate, and glutathione seemed to be non-toxic and also showed positive effects on methanogenic growth (Zhang and Maekawa 1996).

At thermophilic conditions (53°C), the solubility of hydrogen sulphide decreased and consequently the availability of sulphur for methanogens was also lower. From this background, an alternative S-source which should be more stable under thermophilic conditions was investigated.

The following sulphur compounds were tested: Sodium thiosulphate, thioacetic acid (Scherer 1987) und tetrathionate. Each compound (0.05 mM) was added to HAW-medium, from which all S-sources had been excluded.

Table 12 shows a test with various sulphur sources and an inoculum of *Methanothermobacter marburgensis* at 53°C. Gas pressure was measured by a manometer. H_2-CO_2 (80%/20%) was fed up to 2 bar overpressure.

Table 12 Test for an alternative sulphur source with *Methanothermobacter marburgensis* and HAW-medium

Date	(bar)	Na$_2$S		Tetrathionate		Thiosulphate		Thioacetic acid	
	duplicate	a	b	a	b	a	b	a	b
0 d	Pressure	2	2	2	2	2	2	2	2
	Feeding								
3 d	Pressure	0.869	0.690	0.642	0.669	0.666	1.116	0.512	0.753
	Feeding	√	√	√	√	√	√	√	√
5 d	Pressure	1.132	**0.341**	0.921	0.898	0.947	**0.251**	0.823	0.988
	Feeding		√				√		
9 d	Pressure	1.034	**0.437**	0.916	0.887	0.879	**0.598**	0.801	0.963
	Feeding		√				√		

All cultures were fed directly after inoculation up to 2 bar. The test above shows that all test cultures consumed hydrogen very actively within three days, recognised by the reduced pressure below 1.1 bar (3 d). After second feeding, all cultures were still very active, especially the culture with Na$_2$S and thiosulphate, which partially showed a pressure below 0.3 bar (5 d). After third feeding in the cultures with Na$_2$S (b) and thiosulphate (b), they still showed high activity (9 d). In terms of cultivation for *Methanothermobacter marburgensis* under thermophilic conditions, Na$_2$S and thiosulphate was the best option for an alternative sulphur source.

The second test for an alternative sulphur source was performed under mesophilic conditions with the inoculum of *Methanoculleus bourgensis* (table 13).

Table 13 Test for an alternative sulphur source with *Methanoculleus bourgensis and HAW-medium*

Date	(bar)	Na$_2$S		Tetrathionate		Thiosulphate		Thioacetic acid	
	duplicate	a	b	a	B	a	b	a	b
0 d	Pressure	2	2	2	2	2	2	2	2
	Feeding								
3 d	Pressure	1.778	1.946	1.78	1.657	1.665	1.581	1.867	1.945
	Feeding								
5 d	Pressure	0.782	1.824	0.774	0.724	0.464	0.684	1.782	1.823
	Feeding	√	√	√	√	√	√	√	√
9 d	Pressure	**0.934**	2.215	**0.948**	**0.681**	**0.806**	**1.169**	2.246	2.017
	Feeding	√		√	√	√			

All cultures were fed directly after inoculation up to 2 bar overpressure. Unlike the first test with *Methanothermobacter marburgensis*, all cultures, regardless of the sulphur source, showed low activity in three days. In five days all cultures except one culture with thioacetic acid started to consume hydrogen. *M. bourgensis* grew slower than *M. marburgensis* in general. After second feeding, tetrathionate and thiosulphate exihibited more positive effects on the growth of methanogens than others. In terms of cultivation of *M. bourgensis* under mesophilic conditions, tetrathionate and thiosulphate mostly influenced the growth.

Based on the two tests, thiosulphate was chosen as an alternative sulphur source. It should be noticed that all of the chemicals tested above were very odoriferous, especially thioacetic acid, which was extremely fetid.

Cultivation of methanogens in liquid medium

In the beginning, several attempts failed. For instance, after the first inoculation, methanogens could grow well, but after the second and third transfer, methanogens did not grow very often, due to e.g. the lack of some nutrients. After improvement of the medium components (table 10), they grew in transferred media without limiting the growth rate.

Cultivation with liquid medium was generally much easier than that with solid medium. Isolation of target methanogens by this method could be achieved by a specific carbon source and the repetition of the transfer and dilution series. This method with liquid medium did not seem to be sensitive enough to selectively isolate various or new target methanogens. In other words, similar methanogens could occur again and be isolated. Consequently, the probability of the isolation of new methanogens became lower. Another problem was the separation of target methanogens from other methanogens in liquid culture. Sometimes, a morphological mono-culture was contaminated by other methanogens over time, e.g. the separation of coccoid-type methanogens from rod-type methanogens. This separation was very difficult and took a lot of time. When coccoid-type (e.g. *M. bourgensis*) methanogens were obtained and grew to a certain density, the culture should be promptly transferred and DNA was extracted from the culture to identify it by genome sequencing. Otherwise, there was a risk of contamination by rod-type methanogens. Generally, rod-type methanogens grew faster than coccoid-type methanogens and were easier to cultivate.

Working under anaerobic conditions without an anaerobic chamber was a challenge due to its significant cost and the demand for space. After many failures, an optimised working process at every step without contact of oxygen was achieved. The exclusion of oxygen from all vessels and media with a vacuum pump was performed successfully. A great amount of cannulas and syringes were used for sterile and anaerobic work. The blue butyl rubber stopper was found to be very stable and gas-tight; it could be used several times (approximately 20-40 uses) for several months.

The developed pressure measurement could successfully replace other measurement methods for the assessment of growth, like turbidity by a spectrometer. Very often, sedimentations were found in the medium during cultivation, which could cause incorrect measurements when using a spectrometer. The pressure measurement was very often performed not only for checking the cell growth but also for proof of the gas-tightness of the vessels. It was a very reliable and useful method for anaerobic cultivation in general. However, it was not suitable for the analysis of growth kinetics, where pressure should be measured several times in a tube containing only 5 mL of medium. At every pressure measurement, the cannula of the manometer was inserted and removed from the tube. During this procedure, a small amount of gas (0.1-0.2 bar depending on the original pressure) could escape.

Proper preparation of the medium was essential but it was a routine and time-consuming work. Normally, 2 x 200 mL (400 mL) medium was freshly made. Each component of the medium beside macro-elements had already been prepared in a separate serum bottle under anaerobic (sterile) conditions. To the best of our knowledge, filtration (filter paper 0.2 μm pore size) with a vacuum pump was the easiest and quickest method for sterilisation of the medium. It was very reliable. This method excluded the possibility of precipitation of some components or inactivation of the heat-unstable components like vitamins.

Cultivation vials used for this study were appropriate for strict anaerobic cultivation. In particular, the Balch-type tube with a blue butyl rubber stopper was very stable, and pressure-resistant. Among all of the vials used, the quickest and densest cell concentration was achieved in the Balch-type tube, probably due to the biggest contact surface between cells and hydrogen gas in terms of the low solubility of hydrogen (0.0014 g H_2/kg H_2O at 37°C, solubility is about thousand times lower than CO_2). The tubes were placed horizontally in an incubator.

After daily checking of the culture microscopically, an enriched culture was transferred and serially diluted. Under flushing with N_2, adding medium as well as inoculating was easily performed, along with a transfer and dilution series by the established equipment in this study. During this step, the medium normally did not become pink because of the added resazurin, which was a very sensitive redox indicator. As is already known, transfer and dilution series are the most frequently used methods for isolation in a liquid medium (Cheng et al. 2011; Mori et al. 2012). After several transfers (7–10 times) or direct dilution series (10^{-1} to 10^{-3}) from the starter culture, the dominating culture could be enriched and isolated. At higher dilutions of more than 1000 times, the cultures often did not grow or it might take long time to let them grow.

As a carrier material for stabilisation (immobilisation) of methanogenic growth, pellets of borosilicate glass (1.5 mm) and alginic acid were tested (Scherer et al. 1981). However, this test failed due to the level of oxygen inside the porous glass and alginic acid.

Additionally, three variables for promoting the growth of *Methanoculleus*, which is generally slow-growing, were tested: the addition of trace elements, addition of the medium (1:1), and transfer to new medium without allowing rod-type methanogens to occur. In this study, the third method gave the best results and the mono-culture was harvested for DNA-analysis before the occurrence of rod-type methanogens.

Cultivation of methanogens on solid medium

All samples used for this study (table 2) were inoculated several times on solid medium with different methods (figure 11). Most attempts failed probably due to oxygen toxicity or change of pH value by Na_2S added. According to Scott at al. (1983), methanogens from rumen could be inhibited by dissolved oxygen concentration below 30 nM (1 ppm). Although a little amount of the oxygen was reduced by reducing agents, it might be not enough to remove oxygen in solid medium unlike complete reduction of oxygen in liquid medium (maximum solubility of the oxygen at 25°C is 8 mg/L (0.25 mM), 1 mM Na_2S could theoretically reduce the total dissolved oxygen). Pure N_2 (99.995%) was used for making anoxic condition in this study, but it seemed to be not pure enough because 0.005% (50 ppm) of air would result in approximately 10 nM of dissolved oxygen under 1 bar and at 37°C according to **Henry-Dalton's law**. It was not clear that such little amount of oxygen might inhibit the methanogens. After sufficient impact time (1-2 days) of Na_2S (1 mM) pink color of the solid medium (through resazurin) became clear indicating a complete reduction of the oxygen. Thus the possibility of oxygen toxicity was removed. Another possible factor on cultivation on solid medium would be the pH value. The pH value of a Na_2S solution is approximately 9. After addition of Na_2S to the solid medium, its pH value should get higher which might cause unfavorable conditions for methanogens. To test this assumption, H_2S can be used as a reducing agent instead of Na_2S. All vials should be anaerobically stored in an anaerobic chamber for a few days before use (Jones et al. 1983, Stantscheff et al. 2014). Many types of colonies occurred, but they were not methanogens. Various tests were carried out to identify why methanogens were not growing on the solid medium, e.g. in terms of different concentrations of hydrogen sulphide, oxygen toxicity (increase of reducing agent), water content, and trace elements. However, no clear reason was found during this study. However, two colonies of *Methanothermobacter thermautotrophicus* and one colony of a coccoid-type *Methanothermobacter thermophilus* were obtained in roll-tubes-Furthermore, one colony of *Methanothermobacter thermautotrophicus* in angular agar media and in an anaerobic petri-dish box was obtained. However, unfortunately, they did not grow in a liquid medium again.

Contamination by two actively motile bacteria
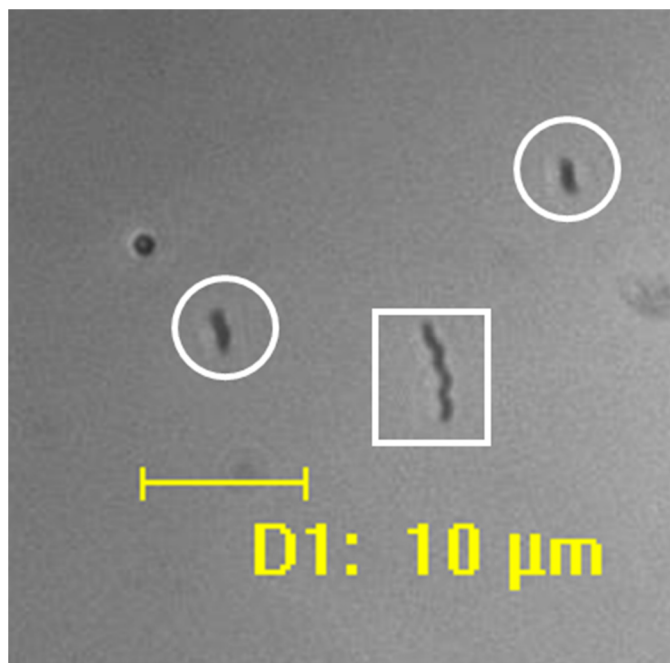
Figure 58 Contamination of enriched cultures by two actively motile bacteria

During the study, two different motile bacteria were a large problem in strict anaerobic cultivation; they were very actively motile. The bacteria in a circle moved bidirectionally in a line or circle. These bacteria were much faster than the longer spiral formed one shown in a square, figure 58. The longer spiral formed one could grow to more than 20 µm. All antibiotics (see method) were used for the removal of these microorganisms, but they were very resistant. These bacteria were contaminated by e.g. a cannula. A characteristic feature of the contaminated culture with the above bacteria was a strongly odoriferous. They did not grow on agar media (EN ISO 6579), which was wrapped (sealed) with parafilm.

Chloramphenicol reduced the presence of the spiral forming species as well as the small one to some extent. A combination of multi-antibiotics like chloramphenicol, bacitracin, cycloserine, cefotaxime, and rifampicin removed the above contaminating bacteria. After autoclaving for 20 min, they were inactivated.

Antibiotics

All used antibiotics inhibited or killed bacteria (not systematically analysed). Interestingly, chloramphenicol also inhibited the growth of a strain of *Methanosarcina und Methanospirillum* in accordance with a study (Hilpert et al. 1981). However,

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Methanoculleus grew well in the presence of chloramphenicol. However, other antibiotics like rifampicin, cycloserine, vancomycin, cefotaxime, cefoxitin, bacitracin, cefsulodin, and ampicillin did not inhibit the used methanogens. All antibiotics (see appendix 7.2) were added and pre-incubated for 1-2 days at 37°C due to instability against temperature. After 1-2 days, the culture was moved to the desired temperature.

Visualization of a grown colony of methanogens
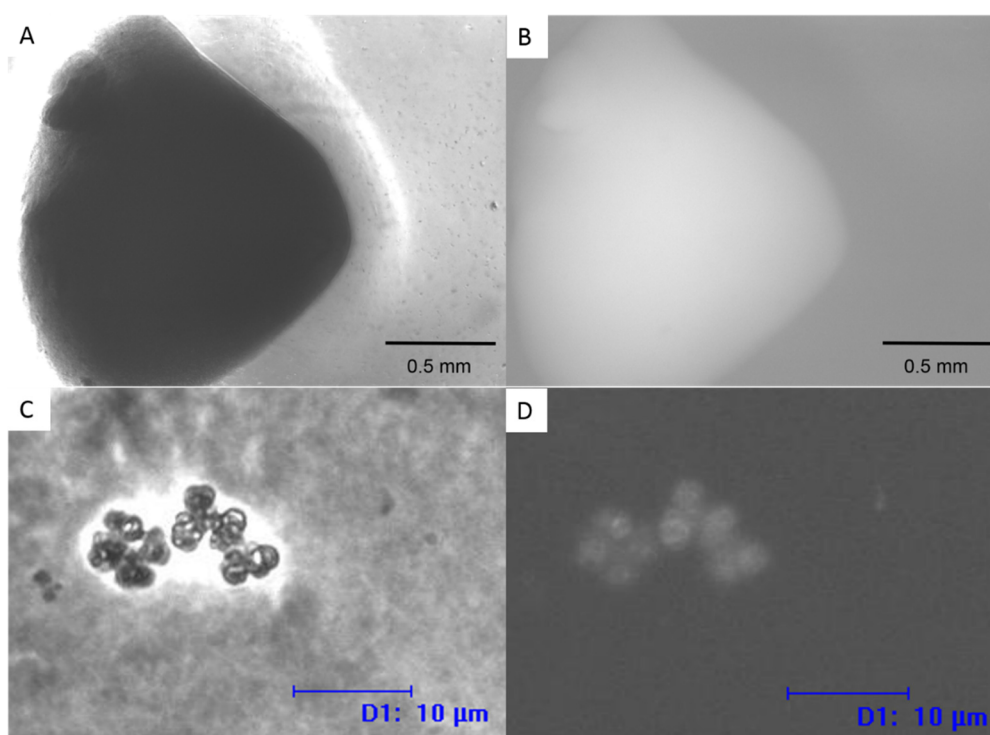
Figure 59 Differentiation of methanogenic colonies from bacterial colonies

Differentiation of a methanogenic colony from other bacterial colonies was necessary (see figure 17). When a colony shines in fluorescence mode, it should be theoretically a methanogenic colony (Doddema und Vogels 1978). A colony was detected in phase contrast mode (A) and fluorescence mode (B) under 40x magnification. The shining colony was picked and analysed again under 400x magnification in phase contrast (C) and in fluorescence mode (D). Thereby a shining *Methanosarcina* (D) was found. Thus, this method for detection of methanogenic colony was confirmed. But this colony contained two types of microorganisms, *Methanosarcina* and rod-type microbe (dominant), which seemed to be a syntrophic consortium.

4.2. Identification of enriched cultures and isolates

During this study, 3 isolates and 7 enriched culture were obtained (table 14). Two or three of isolates were taken for complete genome sequencing analysis after up to 4 µg of DNA was collected. To collect 4 µg of DNA, two or three repetitions of 20 x 10 mL cultivations were performed. Sometimes, coccoid-type methanogens did not grow well under the same conditions. This was a big problem.

A methanogenic coccoid of *Methanoculleus bourgensis* was isolated from a mesophilic biogas fermentor Biowerk fed with biowaste at a relatively high concentration of ammonium (4000–6000 mg/L). In the original culture, mostly coccoid-type methanogens could be seen. After 11 transfers, a monoculture was obtained. However, this grew relatively slowly (3–10 days up to the high density of culture) and could be easily contaminated with other rod-type methanogens. This species was completely sequenced.

Long filament-type methanogens, e.g. *Methanospirillum,* were found in the Biowerk fermentor during the cultivation in mesophilic conditions. Separation of this species from other rod-type methanogens as well as contaminating bacteria failed. In the sample of Biowerk, also *Methanothermobacter thermoautotrophicus* was enriched under thermophilic condition. In the same culture of Biowerk, a rod-type methanogens *Methanobacterium curvum* growing rapidly was highly enriched.

From the Bützberg biogas plant fed with municipal biowaste (percolate of solid state fermentation), a rod-type methanogen was isolated under mesophilic conditions. This isolated *Methanobacterium curvum* was completely sequenced by a partner Cebitec.

From the agricultural biogas plant Ernesto in Italy, a rod-type methanogen *Methanothermobacter thermoautotrophicus* was enriched under mesophilic and thermophilic conditions.

From the fermentor "Hocke", mini-coccoid-type methanogens were enriched. This original culture contained two types of methanogens (rod and coccoid). This culture was transferred to a new medium at 26°C and afterwards transferred again into a new medium at 53°C. Finally, a mini-coccoid-type methanogen was obtained. However, this culture was later contaminated by bacteria (figure 58). After transfer with antibiotics several times, a monoculture was obtained again. Interestingly, *Methanosarcina mazei* occurred as a single coccoid type, not as a multicellular packet (Boone and Mah 1987). This seemed to be the first finding of *Methanosarcina mazei* in a biogas plant.

The rod-type methanogen *Methanothermobacter marburgensis* was relatively simply isolated from the thermophilic farmer biogas plant Viersen after 6 transfers. It seemed to be the first isolate of *M. marburgensis* from a biogas fermentor, which was originally isolated from a sewage sludge plant in Marburg in Germany (Liesegang et al. 2010). This species was given to the project partner for total genome sequencing.

Table 14 Inoculum and obtained enriched cultures and isolates

Source (plant name)	Substrate of plant, Temperature	Nucleotide	Obtained culture (cultivation temperature)	Similarity (%)	Antibiotics used (Appendix 7.2)	Note
Bavarian-LfL	Grass, 37°C	475	*Methanosarcina barkeri* CM1 – CP008746 (37°C). Highly enriched.	99.8	4, 5, 9	Multicellular packets
Biowerk	Food waste, 41°C	471	*Methanoculleus bourgensis* MS2T – HE964772 (37°C). **Isolated**.	100	1, 7, 8	Cocci. Completely sequenced.
		471	*Methanospirillum stamsii* str.sp – NR_117705 (37°C). Enriched.	98.9		Filament
		476	*Methanothermobacter thermoautotrophicus* - Delta H – NR_074260 (53°C). Enriched.	100		Rod
		1000	*Methanobacterium curvum* - GU129101 (37°C). Highly enriched.	99.7		Rod
Bützberg	Food waste, 37°	475	*Methanobacterium curvum* – GU129101 (37°C). **Isolated**.	100	2, 5, 8	Rod. Completely sequenced
Dario	Maize, triticale, 41°C	471	*Methanoculleus bourgensis* MS2T – HE964772 (26°C). Enriched.	100	4, 7, 8	Coccoid
Ernesto	Maize, triticale, 41°C	476	*Methanothermobacter thermoautotrophicus* - Delta H – NR_074260 (37°C). Enriched.	100	7, 8	Rod
Hocke	Cattle manure, maize, 40°C	475	*Methanosarcina mazei* – CP009514 (53°C). Enriched.	100	1, 7, 8	Mini cocci
Viersen	Pig manure, maize, 53°C	476	*Methanothermobacter marburgensis* – CP001710 (53°C). **Isolated.**	100	3, 7, 8	Rod. Sequenced.

Picture examples of isolates

Methanothermobacter marburgensis isolated in this study from the biogas plant Viersen is shown in figure 60. It was a rod-type methanogen with a length of approximately 2.5–6 µm (A). Cells were not motile, but fluorescent at a excitation wavelength of 420 nm (B).
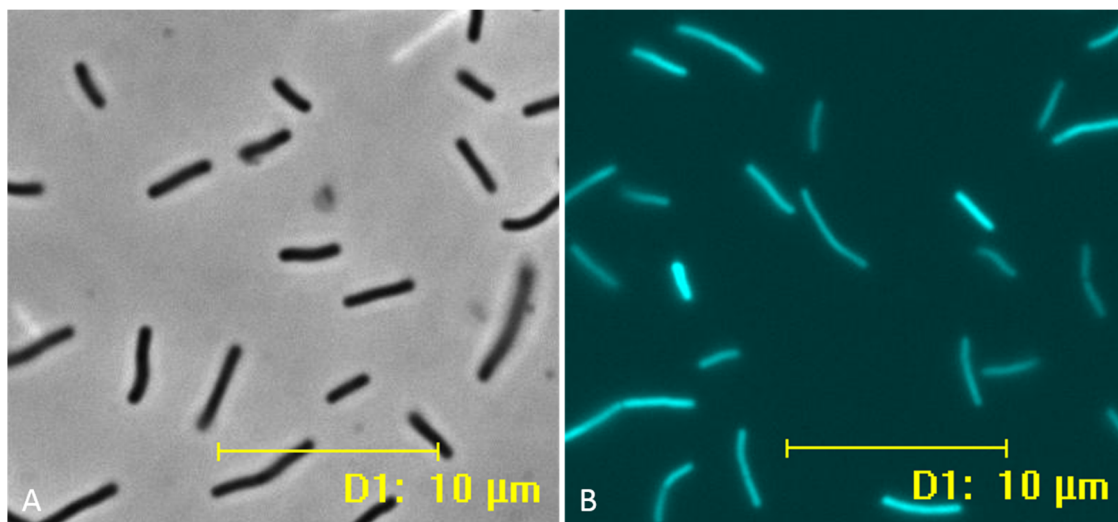
Figure 60 *Methanothermobacter marburgensis* **isolated from the farmer biogas plant Viersen**

Methanoculleus bourgensis isolated in this study is shown in figure 61. It was a coccoid-type methanogen and the diameter of the species was approximately 1.5–2.5 µm (A). Cells were not motile, but fluorescent at a wavelength of 420 nm (B).
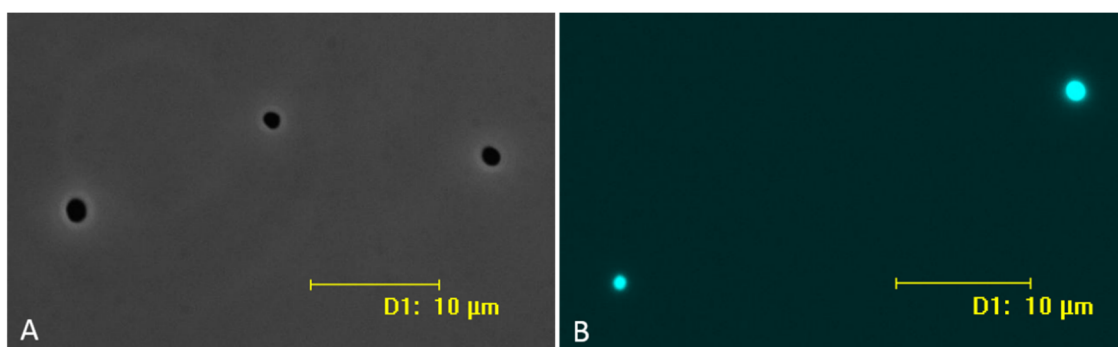
Figure 61 *Methanoculleus bourgensis* **isolated from the biogas plant Biowerk**

Furthermore, filament-type methanogens (*Methanospirillum stamsii*) from the Biowerk biogas plant. Multicellular packet-type methanogens were also isolated from a biogas plant fed simply with grass.

Chapter 5: Conclusion

It's not that I'm so smart, it's just that I stay with problems longer

Albert Einstein

5. Conclusion

The aim of the study was the development and application of rapid methods for the quantification and cultivation of methanogens in bio-methane producing fermentors. To realise this aim, an extensive literature research was performed and critically discussed. Along the literature research, numerous tests were carried out for the optimisation of each step. Many research questions and problems during optimisation of the techniques occurred which were tried to be solved or presented as results and discussion. The established methods in this study were presented in the methods section. In particular, the developed microscopic technique was sufficiently validated and optimised. Furthermore, the microscopic technique was applied in many large-scale fermentors and lab-scale reactors for microbial analyses to identify a new relationship between microscopic observation and operational parameters. The microscopic technique has been presented at national and international conferences as poster and oral presentations, as well as in the format of a journal paper (appendix 7.4), in order to prove and discuss the technique. By using this microscopic technique, it was first found that morphological changes of methanogens according to operational parameters like temperature or ammonia perturbation could play a role as an early indicator. Furthermore, a quantitative microscopic fingerprinting index (QMFI) was developed and to some extent validated as an application tool during this study. For this, tests and observations of about 50 large-scale biogas plants and lab-scale reactors over approximately 2 years were performed. It was an empirical value based on microscope and chemical analysis data. This QMFI was the first approach combining microscopic quantification data with chemical data to estimate the bioprocess of anaerobic digesters more thoroughly. However it was not possible to find enough scientific evidence and explanation (references) for establishment of the new QMFI which is a challenge left for the future. The QMFI is a suggested microbial indicator for a complicated biological system, which was totally based on a long term observation including extensive data under microscope. Thus, it was not always comprehensible and partially very intuitive.

An extensive molecular based analysis showed that numerous microorganisms in anaerobic digesters could not be classified (Kröber et al. 2009). There were not assignable data. From this point of view, isolation and characterization of new methanogens are very important. To understand the methanogens of anaerobic digesters, a simple and rapid method for the cultivation and isolation of methanogens was searched and found, without using a cost-intensive anaerobic chamber. The main target methanogens were H_2-CO_2-

consuming hydrogenotrophic methanogens. Hence, all vials had to be pressure-resistant up to 2 bar overpressure. Since methanogens are strict anaerobic *Archaea*, all procedures should be performed under strictly anaerobic conditions, as well as with the sufficient exclusion of oxygen from all media. Simultaneously, all materials should be sterilely treated in order to obtain an isolate as a pure culture. Since methanogens are very ambitious *Archaea* and can only consume a very limited selection of substrates, the medium should be optimised precisely by testing different reference media (DSM 141, 119, 287, 318, and 332) and reference methanogens (DSM1498, DSM3915, DSM3267, and DSM 2133). Numerous cultivation tests including the transfer and dilution steps in liquid medium were performed and several isolates were obtained, e.g. *Methanothermobacter marburgensis* and *Methanoculleus bourgensis*. Nine antibiotics were used daily to remove untargeted bacteria e.g. vancomycin, rifampicin, bacitracin, cycloserine, and cefotaxime. In order to identify new methanogens and to isolate methanogenic colonies on solid medium, numerous attempts of cultivation were carried out with three different types of vials, as an angular agar medium tube, a roll-tube, and an anaerobic petri-dish box. The last technique, the anaerobic petri-dish box, was developed during the study, which was gas-tight up to 2.5 bar overpressure and enabled the easy detection and picking of methanogenic colonies. However, isolation of new methanogens using a solid medium failed in this study, although numerous different types of bacterial colonies were found. There was a significant problem of contamination by two actively motile bacteria which were normally very resistant to antibiotics. It was found that a combination of multi-antibiotics like chloramphenicol, bacitracin, cycloserine, cefotaxime, and rifampicin removed these contaminating bacteria. Another difficulty was separation of the target methanogens from non-target methanogens e.g. coccoid-type methanogens from rod-type methanogens. To solve this problem, transfer and addition of NaCl, MgCl, and NH_4Cl as well as cultivation at lower temperature would be helpful. The established method and knowledge for cultivation and isolation in this study without an anaerobic chamber could be useful for a universal application without any risk of explosion and cost.

However, there remains many uncompleted work regarding cultivation of methanogens. Obtained isolates were not further characterised due to time limitation. Methanogenic volumetric activity of the isolates was not determined. Effect of environmental conditions on physiology of isolates, e.g. morphology was not systematically investigated. Investigation with pure cultures may support the hypothesis of the QMFI in the future,

which is actually the expected synergy effect as a formulated objective of this study (figure 3).

In this study, microscopic quantification was optimised and validated. Furthermore, application of this microscopic technique was developed. On the other hand, a simple cultivation and isolation tool for methanogens was established, optimised and developed. Simple tools for the quantification and isolation of methanogens should contribute to creating new aspects of monitoring for the analysis of microbial quality of biogas plants and to identify new methanogens for the improvement of scientific knowledge of anaerobic digestion.

New (novel) findings through this work were summarised as following:

- ➢ It was firstly suggested that measurement of the relative auto-fluorescence intensity (%) of methanogens based on their coenzyme F_{420} was feasible by a fluorescence microscope including an image analysis software. It has been recognized as a reliable parameter during measurement of more than 200 samples. It could be used to relate to activity of methanogenesis (see section 3.1. and 3.4.).

- ➢ It was firstly suggested that morphological change of methanogens could be used as a process parameter e.g. for early warning indicator as well as a rough estimate for monitoring of methanogenic population, i.e. morphological classification (see table 6 and figure 37; Kim et al. 2014).

- ➢ The developed microscope analysis revealed that quantification of methanogenic diversity by molecular analysis like qPCR or metagenomics could not always be in accordance with the actual state of methanogenic community. It was firstly proposed that dual analysis of microscope and molecular-based methods was necessary for accurate quantification of methanogenic population. The results will be published.

- ➢ The pre-treatment of environmental samples for microscopic quantification of microbes was established and validated (section 3.1. and 3.2.). The results will be published.

- ➢ Anaerobic Petri-Dish box for cultivation of hydrogenotrophic methanogens on solid-media under overpressure condition was developed (see figure 12).

- ➢ A new concept for measurement of the health of biogas reactor was empirically created, 'quantitative microscopic fingerprinting index' (QMFI). It consists of methanogenic cell data, chemical habitat data in environment, and biodiversity of microbes linking the microbes and the environment (see section 3.4).

Some future works are listed as following:

➢ Some vague parts of the QMFI should be supplemented by scientific evidences.

➢ The QMFI should be validated with extensive analysis data. For instance, the QMFI from a pure culture of a methanogen displaying a high methanogenic volumetric activity and the QMFI from the same pure culture of the methanogen showing a bad methanogenic volumetric activity have to be compared.

➢ Extensive morphological classification should be performed and validated with molecular-based analysis on the basis of pure cultures.

➢ The isolates obtained in this study should be analysed by using total genome sequencing (it will be carried out by a partner).

➢ The isolates should be characterised, especially with the developed microscope technique.

➢ The isolates should be investigated for possibility of bioaugmentation or application as a pure culture for biological methanation.

➢ Analysis with a pure culture by changing environmental conditions should be accompanied to support the findings by the developed microscope technique.

➢ Most of all, the basic for all around microbial experiments is characterization of microbes as pure cultures. A major obstacle to obtain pure culture is still the difficulty of cultivation and isolation technique of target microbes from environment. It is very time-consuming and laborious works. During microscopy analysis, various morphotypes of methanogens have been observed. But it often failed to isolate them. To date, extensive molecular-based analyses have been developed and performed in the area of anaerobic digestion, but not on the basis of pure cultures. Without argumentation by characterization of the pure cultures, the results of molecular-based analyses may be doubtful. At least, microscope analysis should be accompanied to the molecular-based analyses.

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7. Appendix

7.1.   The developed macros for quantification and classification of microorganisms by Image Pro 7

The process logics by Image Pro 7

```
Sub batch_process_Kim()
 While fName <> ""
        ' Print out the file name and its attributes
            Debug.Print GetAttr(gDirStart + fName); " "; fName
            ' Load the image
            docID = IpWsLoad(gDirStart + fName, "")
            ' Don't process if there is a failure loading
            If docID >= 0 Then
                                    If script_domain = "fluo_count" Then
                                    Call Fluo_Kim()
                                    ElseIf script_domain = "fluo_shape" Then
                                    Call FLUO_SHAPE_Kim()
                                    ElseIf script_domain = "fluo_rod" Then
                                    Call FLUO_ROD_Kim()
                                    ElseIf script_domain = "fluo_methanogen_count" Then
                                    Call Fluo_Methanogen_Kim()
                                    ElseIf script_domain = "fluo_methanogen_shape" Then
                                    Call FLUO_methanogen_SHAPE_Kim()
                                    ElseIf script_domain = "fluo_methanogen_rod" Then
                                    Call FLUO_methanogen_ROD_Kim()
                                    ElseIf script_domain = "fluo_methanogen_coccoid" Then
                                    Call FLUO_methanogen_coccoid_Kim()
                                    End If
                                    ret = IpDcUpdate(DC_FETCH)
                Else
                            Debug.Print "Error loading "; gDirStart + fName
                End If
        ' Get the next file name
        fName = Dir()
    Wend
        Call excel_output_Kim()
        ' ret = IpMacroStop("All images in directory processed.", MS_MODAL)
```

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End Sub

The filtration and measurement parameters for total cells by Image Pro 7

```
Sub Fluo_Kim()
ret = IpSCalShow(0)
        ret = IpSCalSelect("400x Kim")
        ret = IpSCalSetLong(SCAL_CURRENT_CAL, SCAL_APPLY, 0)
   ret = IpFltShow(0)
   ret = IpFltGauss(7, 1, 1)
        ret = IpBlbShow(1)
        ret = IpFltFlatten(1, 5)
   ret = IpFltOpen(MORPHO_2x2SQUARE, 1)
        ret = IpFltConvolveKernel("HIGAUSS.7x7", 10, 2)
   ret = IpBlbSetAttr(BLOB_AUTORANGE, 1)
        ret = IpBlbSetAttr(BLOB_BRIGHTOBJ, 1)
        ret = IpBlbSetAttr(BLOB_MEASUREOBJECTS, 1)
   ret = IpBlbSetAttr(BLOB_8CONNECT,0)
        ret = IpBlbSetAttr(BLOB_LABELMODE,0)
        ret = IpBlbSetAttr(BLOB_OUTLINEMODE,1)
        ret = IpBlbSetAttr(BLBSET_OUTLINECOLOR, 1)
        ret = IpBlbSetAttr(BLOB_SMOOTHING,3)
        ret = IpBlbSetAttr(BLOB_FILLHOLES,1)
        ret = IpBlbSetAttr(BLOB_CLEANBORDER,0)
        ret = IpBlbSetAttr(BLOB_FILTEROBJECTS, 1)
        ' ret = IpBlbSetAttr(BLOB_MINAREA,1)
        ret = IpBlbEnableMeas(BLBM_AREA, 1)
        ret = IpBlbSetFilterRange(BLBM_AREA + CALIB_UNIT, 0.1, 100.0)
        ret = IpBlbEnableMeas(BLBM_MEANFERRET, 1)
   ret = IpBlbSetFilterRange(BLBM_MEANFERRET + CALIB_UNIT, 0.1, 300.0)
   ret = IpBlbEnableMeas(BLBM_PERAREA, 1)
   ret = IpBlbEnableMeas(BLBM_LENGTH, 1)
   ret = IpBlbSetFilterRange(BLBM_LENGTH + CALIB_UNIT, 0.2, 300.0)
   ret = IpBlbEnableMeas(BLBM_WIDTH, 1)
   ret = IpBlbSetFilterRange(BLBM_WIDTH + CALIB_UNIT, 0.1, 100.0)
   ret = IpBlbEnableMeas(BLBM_MINRADIUS, 1)
   ret = IpBlbEnableMeas(BLBM_MINORAX, 1)
   ret = IpBlbSetFilterRange(BLBM_MINORAX + CALIB_UNIT, 0, 3.5)
   ret = IpBlbEnableMeas(BLBM_PERIMETER, 1)
```

```
ret = IpBlbSetFilterRange(BLBM_PERIMETER + CALIB_UNIT, 1, 500)

ret = IpBlbEnableMeas(BLBM_DENSITY, 1)

ret = IpBlbSetFilterRange(BLBM_DENSITY + CALIB_UNIT, 1, 255.0)

ret = IpBlbEnableMeas(BLBM_ROUNDNESS, 1)

ret = IpBlbSetFilterRange(BLBM_ROUNDNESS + CALIB_UNIT, 1, 100.0)

If script_domain = "fluo_count" Or script_domain = "fluo_shape" Then

ret = IpBlbEnableMeas(BLBM_ASPECT, 1)

ret = IpBlbSetFilterRange(BLBM_ASPECT + CALIB_UNIT, 0.0, 100.0)

If script_domain = "fluo_shape" Then

ret = IpBlbSetAttr(BLOB_OUTLINEMODE,5)

End If

    ElseIf script_domain = "fluo_rod" Then

ret = IpBlbEnableMeas(BLBM_ASPECT, 1)

ret = IpBlbSetFilterRange(BLBM_ASPECT + CALIB_UNIT, 1.5, 100.0)

ret = IpBlbSetAttr(BLOB_OUTLINEMODE,5)

End If

' ret = IpBlbMeasure()

    ret = IpBlbCount()

End Sub
```

The classification parameters for total cells by Image Pro 7

```
Sub FLUO_SHAPE_Kim()

    Call Fluo_Kim()

    ipClassifiers(0) = BLBM_ASPECT

    ' ret = IpBlbShowAutoClass(ipClassifiers(0), 1, 2, 1, 1)

  ipBins(0) = 0.0

    ipBins(1) = 1.5

    ipBins(2) = 3

    ipBins(3) = 100.0

    ret = IpBlbShowSingleClass(BLBM_ASPECT, ipBins(0), 3, 1)

End Sub


Sub FLUO_ROD_Kim()

    Call Fluo_Kim()

  ipBins(0) = 0.0

    ipBins(1) = 1

    ipBins(2) = 7.0

    ipBins(3) = 15.0
```

```
        ipBins(4) = 200.0
        ret = IpBlbShowSingleClass(BLBM_LENGTH, ipBins(0), 4, 1)
```

The command for counting and classifying the total cells by Image Pro 7

```
Sub auto_fluo_count_Kim()
        Call select_dir_YSS()
        Call data_collect_count_Kim()
        script_domain = "fluo_count"
        ret = IpDcShow(1)
        ret = IpDcSet(DC_AUTO, 0)
        Call batch_process_Kim()
End Sub


Sub auto_fluo_shape_Kim()
        Call select_dir_YSS()
        Call data_collect_shape_Kim()
        script_domain = "fluo_shape"
        ret = IpDcShow(1)
        ret = IpDcSet(DC_AUTO, 0)


        Call batch_process_Kim()
End Sub


Sub auto_fluo_rod_Kim()
        Call select_dir_YSS()
        Call data_collect_rod_Kim()
        script_domain = "fluo_rod"
        ret = IpDcShow(1)
        ret = IpDcSet(DC_AUTO, 0)
        Call batch_process_Kim()
End Sub
```

The data scheme by Image Pro 7

```
Sub data_collect_count_Kim()
        ret = IpDcShow(1)
        ret = IpDcUpdate(DC_RESET)
        ret = IpDcUnSelect("<all>","",0)
        ret = IpDcSelect("Image", "Name", 0)
```

```
        ret = IpDcSelect("Count_Size", "Count", 0)
        ret = IpDcSelect("Count_Size", "BLBM_AREA", 3)
        ret = IpDcSelect("Count_Size", "BLBM_Density", 3)
        ret = IpDcSelect("Count_Size", "BLBM_AREA", 2)
        ret = IpDcSelect("Count_Size", "BLBM_AREA", 7)
        ret = IpDcSelect("Count_Size", "BLBM_AREA", 4)
        ret = IpDcSelect("Count_Size", "BLBM_Density", 2)
        ret = IpDcSelect("Count_Size", "BLBM_Density", 4)
End Sub


Sub data_collect_shape_Kim()
        ret = IpDcShow(1)
        ret = IpDcUpdate(DC_RESET)
        ret = IpDcUnSelect("<all>","",0)
        ret = IpDcSelect("Image", "Name", 0)
        ret = IpDcSelect("Count_Size", "Count", 0)
        ret = IpDcSelect("Count_Size", "Classes", 1)
        ' ret = IpDcSelect("Count_Size", "Classes", 2)
    ret = IpDcSelect("Count_Size", "BLBM_LENGTH", 3)
        ret = IpDcSelect("Count_Size", "BLBM_ASPECT", 3)
        ret = IpDcSelect("Count_Size", "BLBM_WIDTH", 3)
End Sub


Sub data_collect_rod_Kim()
        ret = IpDcShow(1)
        ret = IpDcUpdate(DC_RESET)
        ret = IpDcUnSelect("<all>","",0)
        ret = IpDcSelect("Image", "Name", 0)
        ret = IpDcSelect("Count_Size", "Count", 0)
        ret = IpDcSelect("Count_Size", "Classes", 1)
        ' ret = IpDcSelect("Count_Size", "Classes", 2)
    ret = IpDcSelect("Count_Size", "BLBM_Density", 3)
    ret = IpDcSelect("Count_Size", "BLBM_Area", 3)
        ' ret = IpDcSelect("Count_Size", "BLBM_WIDTH", 3)
End Sub
```

The filtration and measurement parameters for methanogens by Image Pro 7

Fluo_Methanogen_Kim()

```
ret = IpSCalShow(0)
        ret = IpSCalSelect("400x Kim")
        ret = IpSCalSetLong(SCAL_CURRENT_CAL, SCAL_APPLY, 0)
    ret = IpFltShow(0)
    ret = IpFltGauss(7, 5, 1)
        ret = IpBlbShow(1)
    ret = IpFltFlatten(1, 10)
    ret = IpFltOpen(MORPHO_2x2SQUARE, 1)
        ret = IpFltConvolveKernel("HIGAUSS.7x7", 10, 2)
    ret = IpBlbSetAttr(BLOB_AUTORANGE, 1)
        ret = IpBlbSetAttr(BLOB_BRIGHTOBJ, 1)
        ret = IpBlbSetAttr(BLOB_MEASUREOBJECTS, 1)
    ret = IpBlbSetAttr(BLOB_8CONNECT,0)
        ret = IpBlbSetAttr(BLOB_LABELMODE,1)
        ret = IpBlbSetAttr(BLOB_OUTLINEMODE,1)
        ret = IpBlbSetAttr(BLBSET_OUTLINECOLOR, 1)
        ret = IpBlbSetAttr(BLOB_SMOOTHING,3)
        ret = IpBlbSetAttr(BLOB_FILLHOLES,1)
        ret = IpBlbSetAttr(BLOB_CLEANBORDER,0)
        ret = IpBlbSetAttr(BLOB_FILTEROBJECTS, 1)
        ' ret = IpBlbSetAttr(BLOB_MINAREA,1)
        ret = IpBlbEnableMeas(BLBM_AREA, 1)
        ret = IpBlbSetFilterRange(BLBM_AREA + CALIB_UNIT, 0.1, 100.0)
        ret = IpBlbEnableMeas(BLBM_MEANFERRET, 1)
    ret = IpBlbSetFilterRange(BLBM_MEANFERRET + CALIB_UNIT, 0.2, 300.0)
    ret = IpBlbEnableMeas(BLBM_PERAREA, 1)
    ret = IpBlbEnableMeas(BLBM_LENGTH, 1)
    ret = IpBlbSetFilterRange(BLBM_LENGTH + CALIB_UNIT, 0.2, 20.0)
    ret = IpBlbEnableMeas(BLBM_WIDTH, 1)
    ret = IpBlbSetFilterRange(BLBM_WIDTH + CALIB_UNIT, 0.1, 100.0)
    ret = IpBlbEnableMeas(BLBM_MINRADIUS, 1)
    ret = IpBlbEnableMeas(BLBM_MINORAX, 1)
    ret = IpBlbSetFilterRange(BLBM_MINORAX + CALIB_UNIT, 0, 4.5)
    ret = IpBlbEnableMeas(BLBM_PERIMETER, 1)
    ret = IpBlbSetFilterRange(BLBM_PERIMETER + CALIB_UNIT, 1, 500)
    ret = IpBlbEnableMeas(BLBM_DENSITY, 1)
    ret = IpBlbSetFilterRange(BLBM_DENSITY + CALIB_UNIT, 1, 255.0)
    ret = IpBlbEnableMeas(BLBM_ROUNDNESS, 1)
```

```
ret = IpBlbSetFilterRange(BLBM_ROUNDNESS + CALIB_UNIT, 1, 100.0)
If script_domain = "fluo_methanogen_count" Or script_domain = "fluo_methanogen_shape"
Then
ret = IpBlbEnableMeas(BLBM_ASPECT, 1)
ret = IpBlbSetFilterRange(BLBM_ASPECT + CALIB_UNIT, 1.0, 100)
ret = IpBlbSetAttr(BLOB_OUTLINEMODE,5)
ElseIf script_domain = "fluo_methanogen_shape" Then
ret = IpBlbSetAttr(BLOB_OUTLINEMODE,5)
ElseIf script_domain = "fluo_methanogen_rod" Then
ret = IpBlbEnableMeas(BLBM_ASPECT, 1)
ret = IpBlbSetFilterRange(BLBM_ASPECT + CALIB_UNIT, 1.5, 100.0)
ret = IpBlbSetAttr(BLOB_OUTLINEMODE,5)
ElseIf script_domain = "fluo_methanogen_coccoid" Then
ret = IpBlbEnableMeas(BLBM_ASPECT, 1)
ret = IpBlbSetFilterRange(BLBM_ASPECT + CALIB_UNIT, 1.0, 1.5)
ret = IpBlbSetAttr(BLOB_OUTLINEMODE,5)
End If
ret = IpBlbCount()
End Sub
```

The classification parameters for total cells by Image Pro 7

```
Sub FLUO_methanogen_SHAPE_Kim()
Call Fluo_Methanogen_Kim()
ipClassifiers(1) = BLBM_ASPECT
ipBins(0) = 1.0
ipBins(1) = 1.50
ipBins(2) = 3.0
ipBins(3) = 100.0
ret = IpBlbShowSingleClass(1, ipBins(0), 3, 1)


Sub FLUO_methanogen_ROD_Kim()
Call Fluo_Methanogen_Kim()
ipBins(1) = 1
ipBins(2) = 6.0
ipBins(3) = 15.0
ipBins(4) = 200.0
ret = IpBlbShowSingleClass(27, ipBins(0), 4, 1)
```

Excel output by Image Pro 7

Sub excel_output_Kim()

       ret = IpDcSaveData(gDirStart + script_domain + ".xls", S_Y_AXIS + S_X_AXIS)

End Sub

7.2.   Chemicals

Polyphosphate (100 mL stock solution. p source)

Ingredients	Molar mass Each [g/mol]	Molar mass [g/mol]	Stock solution [g/L] (1g/10mL)	Concentration in stock solution [mM]	Concentration in medium [µM] (1:2000. 0.1mL in 200mL medium)	ppm in medium
$(NaPO_3)_x$	22.9/31/16	101.96x	10g/100mL	1000mM	0.5 mM	15.5 (P) ppm

Obtained by Riedel – de Haën, 50690, Germany

NaHCO₃-Solution (100 mL stock solution. Additional CO₂ source)

Ingredients	Molar mass CO_3 [g/mol]	Molar mass [g/mol]	Stock solution [g/L]. 8.4g in 100mL	Concentration in stock solution [M]	Concentration in medium (1:200) [mM]	ppm
$NaHCO_3$	60.0	84.01	84.01	1	5	300 (CO_3)

Imidazole-Solution (100 mL stock solution. pH-Buffer)

Ingredients	Molar mass [g/mol]	Stock solution [g/100mL]	Concentration in stock solution [M]	Concentration in medium (1:100) [mM]. 2mL in 200 mL medium	ppm
Imidazole	68.08	6.81	1	10	680
HCL25%	36.545	Ca. 6mL			

Filtered by activated carbon filter and oxygen excluded, *obtained by J.T.Baker, UN3263, Deventer, Netherlands*

Macroelement solution (1L stock solution)

Ingredients	Molar mass. each [g/mol]	Molar mass [g/mol]	Stock solution [g/L]	Concentration in medium [mM]	ppm in medium
$MgCl_2*6H_2O$	24.3 Mg	203.3	1	5	121.25 Mg
NaCl	23 Na	58.44	1.75	30*	1034 Na
NH_4Cl	18 NH4	53.49	1	18.6	335 NH_4
$CaCl_2*2H_2O$	40.1 Ca	147.02	73.5 mg	0.5	20 Ca
KCl	39.1 K	74.55	4.78	64.1	2500 K

*max. 40-50mM sodium in medium. Otherwise inhibition possible. Including sodium acetate, sodium formate, Na_2S. sodium polyphosphate

Appendix

Sodium acetate solution (100 mL stock solution)

Ingredients	Molar mass [g/mol]	Stock solution [g/L]	Concentration in Stock solution [mM]	Concentration in Medium [mM] (1:100)	ppm in Medium
$C_2H_3NaO_2$ (Na-Acetate)	82.03	41	500	5	295 Acetate

Autoclaved

Sodium formate solution (100 mL stock solution)

Ingredients	Molar mass [g/mol]	Stock solution [g/L]	Concentration in stock solution [M]	Concentration in medium [mM] (1:100)	ppm in medium
HCOONa	68.01	68	1	10	460 formate

Autoclaved

Resazurin solution (100 mL stock solution) (Scherer u. Kneifel, 1983)

Ingredients	Molar mass [g/mol]	Stock solution [mg/100mL]	Concentration in stock solution [mM]	1:500. 400µl in 200mL medium	Concentration in Medium [µM]	ppm in Medium
Resazurin	257.17	100	4	400 µL	8	2.06

Autoclaved, obtained by SERVA, 34226, Heidelberg, Germany

Appendix

Trace-element solution (100 mL stock solution)

Ingredients	Molar mass each [g/mol]	Molar mass [g/mol]	Stock solution [mg/L]	Concentration in stock solution [μM]	Concentration in medium [μM] (1:100)	ppb in medium
$(NaPO_3)_x$	22.9/31/16	101.96x	10g/L	102mM	1mM	31 P ppm
$ZnCl_2$	65.4 Zn	136.28	6.81	50	0.5	33 Zn
$MnCl_2*4H_2O$	54.9 Mn	197.91	1.98	10	0.1	5.5 Mn
H_3BO_3	10.8 B	61.83	0.62	10	0.1	1.1 B
$CuCl_2*2H_2O$	63.5 Cu	170.48	1.70	10	0.1	6.4 Cu
$NaWO_4*2H_2O$	183.8 W	329.86	3.30	10	0.1	18.4 W
$CoCl_2*6H_2O$	58.9 Co	237.93	11.89	50	0.5	29.5 Co
$NiCl_2*6H_2O$	58.7 Ni	237.71	11.88	50	0.5	29.3 Ni
$Na_2SeO_3*5H_2O$	78.9 Se	263.01	2.63	10	0.1	7.9 Se
$Na_2MoO_4*2H_2O$	95.9 Mo	241.45	2.41	10	0.1	9.6 Mo
$Fe(III)Cl_3*6H_2O$	55.85 Fe	270.30	270	1000	10	558 Fe

Filtrated

Appendix

Vitamin solution (100 mL stock solution) (Wolin et al. 1963)

Ingredients	Molar mass [g/mol]	Stock solution [mg/500mL]	Concentration in stock solution [μM]	Concentration in medium [μM] (1:100)	ppb in medium
Biotin	244.3	1.0	8.20	0.08	19.54
Folic acid	441.41	1.0	4.53	0.05	22.07
Pyridoxinhydrochlorid	205.64	5.0	48.6	0.49	100.76
Riboflavin	376.37	2.5	13.3	0.13	48.92
Thiamin-HCl	337.27	2.5	14.8	0.15	50.6
Nicotine acid	123.11	2.5	40.6	0.41	50.5
D-Ca-Pantothenate	476.54	2.5	10.5	0.10	47.65
Vitamin B_{12}	1355.4	0.1	0.147	0.0015	2.03
p-Aminobenzoic acid	137.14	2.5	36.5	0.36	49.37
Lipoic acid	206.33	2.5	24.2	0.24	49.52

Filtrated

Fe-Cysteine solution (50 mL stock solution) (Scherer and. Kneifel, 1983)

Ingredients	Molar mass [g/mol]	Stock solution [g/50mL]	Concentration in stock solution [mM]	1:500 for 200 mL medium	Concentration in medium [mM]	Note	ppm in medium
Cysteine-HCl-Monohydrate	175.64	1.87	212.5	400μL	0.425	10μl in 5 mL tube	74 cysteine
Fe(II)citrate	245.95	61 mg	5		0.01		0.558 Fe

Filtrated, obtained by Merck, 1.02839.0100, Darmstadt, Germany

Appendix

Na₂S Solution (100 mL stock solution) (Scherer u. Sahm, 1981)

Ingredients	Molar mass [g/mol]	Stock solution Weight [g/100mL]	Concentration in Stock solution [mM]	1:250. for 200 mL medium	Concentration in medium [mM]	Note	ppm in medium
Na₂S*9H₂O	240	6 (or 0.6g/10mL)	250	800 μL#	1	20μl in 5 mL tube	32 S

Make new solution every month, autoclaved, obtained by Sigma Aldrich, 016-009-00-8, Steinheim, Germany

Titan-Citrate-solution (50 mL stock solution) (Zehnder, 1976)

Ingredients	Molar mass [g/mol]	Stock solution Weight [g/50mL]	Concentration in stock solution [mM]	1:1000. for 200 mL medium	Concentration in medium [mM]	Note	ppm in medium
Tri-Na-citrate*2H₂O	294.1	2.94	200	200 μL	0.2	10μl in 5 mL tube	38.4 Citrate
Titan-III-chloride	154.26	5 mL	83.9		0.083	100μl in 5 mL tube	3.9 Ti

Adjust pH 6.8 with 2mol NaOH, autoclave, oxygen exclusion with N₂, obtained by Merck, 1.10789.0001, Darmstadt, Germany

Thiosulfate solution (100 mL stock solution) (Zehnder, 1976)

Ingredients	Molar mass [g/mol]	Stock solution [g/100mL]	Concentration in stock solution [mM]	n 1:100. for 200 mL medium	Concentration in Medium [mM]	Note	ppm in medium
Na-Thiosulfate-pentahydrate	248.18	0.12	5mM	2 mL	0.05	50μl in 5 mL tube	5.6 Thiosulfate

Appendix

Antibiotics (10mL stock solution) *(Kamagata and Mikami 1991; Ferguson et al. 1983; Harris et al. 1984)*

Nr.	Antibiotics	Stock solution	Storage	Working solution	For 5 mL tube
				in 10mL serum flask	[100 µl, 1:50]
1	Cefoxitin (Roth, 4156.1)	100 mg/mL in water	100µl aliquot at -20°C	100 µl aliquot in 10 mL PBS (=1:100. 1000 µg/mL)	20 µg/mL in medium
2	Cefsulodin (Roth, 4014.1)	20 mg/mL in water	100µl aliquot at-20°C	100 µl aliquot in 10 mL PBS (=1:100. 200 µg/mL)	4 µg/mL in medium
3	Vancomycin (Roth, 0242.1)	100mg/mL in water	100µl aliquot at -20°C	100 µl aliquot in 10 PBS (=1:100. 1000 µg/mL)	20 µg/mL in medium
4	Cycloserin (Roth, CN37.1)	100 mg/mL in * buffer	100µl aliquot at -20°C	100 µl aliquot in 10 mL PBS (=1:100. 1000 µg/mL)	20 µg/mL in medium
5	Cefotaxime (VWR, EHERC11064400)	100 mg/mL in water	100µl aliquot at -20°C	100 µl aliquot in 10 mL PBS (=1:100. 1000 µg/mL)	20 µg/mL in medium
6	Chloramphenicol (Roth, 3886.2)	100 mg/mL in ethanol	100µl aliquot at -20°C	100 µl aliquot in 10 mL PBS (=1:100. 1000 µg/mL)	20 µg/mL in medium
7	Ampicillin (Roth, HP62.1)	250 mg/mL in water	100µl aliquot at -20°C	100 µl aliquot in 10 mL PBS (=1:100. 2500 µg/mL)	50µg/mL in medium
8	Rifampicin (Roth, 4163.1)	100 mg/mL in DMSO	100µl aliquot at -20°C	100 µl aliquot in 10 mL PBS (=1:100. 1000 µg/mL)	20 µg/mL in medium
9	Bacitracin (VWR, EHER10418000)	100 mg/mL in water	100µl aliquot at -20°C	100 µl aliquot in 10 mL PBS (=1:100. 1000 µg/mL)	20 µg/mL in medium

*Note: Sodium carbonate buffer 0.05M: 0.294g $NaHCO_3$ (35mM) + 0.159g Na_2CO_3 (15 mM) in 100 mL sterile water. ca. pH 9.5

7.3.   Additional measurement data

To figure 49: Variation of the quantitative microscopic fingerprinting index (QMFI) and methanogenic cell counts with respect to accumulation of propionic acid

Experimental Week	65	72	82	88	99
Total Cells, [N/mL]	4.83E+10	3.92E+10	4.33E+10	3.07E+10	3.69E+10
Methanogenic Cells, [N/mL]	7.18E+08	5.17E+08	4.50E+08	1.08E+09	9.92E+08
Portion of Methanogens to the Total Cells, %	1.5%	1.3%	1.0%	3.5%	2.7%
Methanogenic Fluo-Intensity, %	60%	51%	57%	47%	51%
Mean Value of Single Cell Area, μm^2	1.38	1.51	1.75	1.69	1.33
Total Methanogenic Cell Power (TMCP), [1/cm] TMCP=Methanogenic Cell NumberxCell AreaxFluo-Intensity	5.87	3.92	4.42	8.54	6.72
Volatile Solid of Fermentation Medium, %	7.3%	6.1%	6.6%	7.2%	6.8%
Volatile Fatty Acid in Fermentor, [mg/L]	406	551	2298	838	1571
Propionic Acid, [mg/L]	128	330	1221	73	254
Degradation rate, $(VS_{input}-VS_{output})/VS_{input}$	42%	51%	48%	43%	46%
Biodiversity of Total Cells (0-1)	0.55	0.576	0.59	0.553	0.52
Biodiversity of Methanogens (0-1)	0.65	0.661	0.66	0.5	0.67
Quantitative Microscopic Fingerprinting Index (QMFI, 0-1)	0.355	0.235	0.203	0.546	0.479
Weekly Organic Loading Rate, [kg/m^3]	630	629.91	591	585.45	585
Electricity Production, [kW]	59	61	63	63	63
Maize	21%	21%	13%	20%	20%
Cattle Manure	65%	65%	70%	70%	70%
Dung	4%	4%	0%	0%	0%
Grass	10%	10%	10%	10%	10%
Whole crop silage	0%		7%		0%
Experimental Week	107	112	115	119	145
Total Cells, [N/mL]	4.25E+10	4.49E+10	3.51E+10	3.16E+10	3.10E+10
Methanogenic Cells, [N/mL]	1.12E+09	1.03E+09	7.51E+08	5.42E+08	1.06E+09
Portion of Methanogens to the Total Cells, %	2.6%	2.3%	2.1%	1.7%	3.4%
Methanogenic Fluo-Intensity, %	40%	35%	35%	49%	66%
Mean Value of Single Cell Area, μm^2	1.53	1.72	1.81	1.38	1.36
Total Methanogenic Cell Power (TMCP), [1/cm] TMCP=Methanogenic Cell NumberxCell AreaxFluo-Intensity	6.79	6.17	4.66	3.6	9.32
Volatile Solid of Fermentation Medium, %	7.5%	8.8%	8.1%	8.1%	7.4%
Volatile Fatty Acid in Fermentor, [mg/L]	1951	4851	7708	3672	475
Propionic Acid, [mg/L]	616	1525	3160	1002	39
Degradation rate, $(VS_{input}-VS_{output})/VS_{input}$	41%	30%	36%	36%	41%
Biodiversity of Total Cells (0-1)	0.563	0.60	0.59	0.586	0.51
Biodiversity of Methanogens (0-1)	0.606	0.47	0.36	0.745	0.73
Quantitative Microscopic Fingerprinting Index (QMFI, 0-1)	0.428	0.292	0.19	0.209	0.616
Weekly Organic Loading Rate, [kg/m^3]	585	601	601	601	617
Electricity Production, [kW]	55	57	59	61	86
Maize	20%	15%	15%	15%	16%
Cattle Manure	71%	69%	69%	69%	68%
Dung	0%	0%	0%	0%	0%
Grass	10%	16%	16%	16%	16%
Whole crop silage		0%	0%		0%

To figure 50: Relationship between QMFI and methanogenic cell counts, volatile fatty acid, and specific biogas production rate in fermentor F2 of the biogas plant Seth

Experimental week	32	37	42	47	49	53	60	63
Total Cells, [N/ml]	3.13E+10	4.41E+10	2.91E+10	3.62E+10	4.41E+10	3.60E+10	1.92E+10	3.27E+10
Methanogenic Cells, [N/ml]	5.15E+08	7.66E+08	3.46E+08	4.60E+08	5.11E+08	4.48E+08	3.22E+08	1.01E+09
Portion of Methanogens to the Total Cells, %	1.6%	1.7%	1.2%	1.3%	1.2%	1.2%	1.7%	3.1%
Methanogenic Fluo-Intensity, %	55%	42%	42%	42%	46%	30%	21%	19%
Mean Value of Single Cell Area, μm^2	1.69	1.77	1.77	1.77	1.65	1.47	3.15	1.98
Total Methanogenic Cell Power (TMCP), 1/cm	4.8	5.7	2.6	3.4	3.5	2.0	2.1	3.8
Volatile Solid of Fermentation Medium, %	6.6%	6.4%	6.3%	6.4%	6.6%	6.7%	5.8%	6.3%
Volatile Fatty Acid, mg/l	630	768	717	835	564	671	1019	361
Degradation rate(VS_{input}-VS_{output})/VS_{input}	55%	57%	58%	57%	56%	55%	61%	57%
Biodiversity of Total Cells (0-1)	0.492	0.467	0.478	0.490	0.536	0.557	0.559	0.568
Biodiversity of Methanogens (0-1)	0.708	0.512	0.521	0.336	0.615	0.516	0.659	0.655
Quantitative Microscopic Fingerprinting Index (QMFI,	0.376	0.442	0.223	0.254	0.234	0.136	0.166	0.339
Specific gas production rate (m^3/kg VS/d)	0.36	0.37	0.34	0.35	0.38	0.32	0.34	0.46

Figure 51: Relationship between specific gas production rate and quantitative microscopic fingerprinting index (QMFI) for a laboratory reactor (RM60) fed with fodder beet

Organic Loading Rate [kgVS/m³/d]	5	7	9	11	13
Hydraulic Retention Time, [d]	14.6	11.8	8.4	8.3	8.7
Biogas Production, [mL/d]	16779	18106	26634	33279	42207
Volumetric Gas Production Rate, [m³/m³/d]	3.20	3.40	4.90	5.55	7.03
Specific Gas Production Rate, [m³/kgVS]	0.54	0.53	0.52	0.55	0.60
Methane content [%]	65%	63%	71%	70%	57%
NH_4-N, [mg/L]	1059	1235	890	797	383
Volatile Fatty Acid, [mg/L]	421	417	544	829	3020
Acetic Acid, [mg/L]	306	347	301	277	399
Propionic Acid, [mg/L]	70	34	159	382	1867
Total Cells, [N/mL]	2.05E+10	2.08E+10	1.98E+10	2.29E+10	2.94E+10
Methanogenic Cells, [N/mL]	1.25E+09	1.00E+09	7.11E+08	1.63E+09	4.72E+09
Portion of Methanogens to the Total Cells, [%]	6.1%	4.8%	3.6%	7.1%	16.1%
Methanogenic Fluo-Intensity, [%]	20%	21%	35%	30%	37%
Mean Value of Single Cell Area, μm^2	1.91	2.05	1.70	1.75	1.68
Total Methanogenic Cell Power (TMCP), [1/cm] TMCP=Methanogenic Cell NumberxCell AreaxFluo-Intensity	4.7	4.2	4.2	8.5	29.0
Volatile Solid of Fermentation Medium, %	1.9%	1.9%	2.0%	1.8%	1.7%
Volatile Fatty Acid in Fermentor, [mg/L]	421	417	544	829	3019
Degradation rate, (VS_{input}-VS_{output})/VS_{input}	61%	62%	67%	71%	72%
Biodiversity of Total Cells (0-1)	0.65	0.65	0.66	0.65	0.64
Biodiversity of Methanogens (0-1)	0.25	0.25	0.25	0.25	0.25
Quantitative Microscopic Fingerprinting Index (QMFI, 0-1)	0.358	0.303	0.253	0.501	0.793

To figure 52: Change of total cells, methanogenic cell counts, and QMFI depending on decrease of the volatile solid content in serial connected digesters of a full-scale biogas plant Lanken

	Digester	Post Digester 1	Post Digester 2	Digestate Storage
Total Cells, [N/mL]	5.31E+10	3.71E+10	2.05E+10	2.00E+10
Methanogenic Cells, [N/mL]	8.26E+09	7.10E+09	3.75E+09	2.77E+09
Portion of Methanogens to the Total Cells, %	16%	19%	18%	14%
Methanogenic Fluo-Intensity, %	31%	27%	26%	23%
Mean Value of Single Cell Area, μm^2	2.39	2.45	2.16	2.07
Total Methanogenic Cell Power (TMCP), [1/cm] TMCP=Methanogenic Cell Number x Cell Area x Fluo-Intensity	62.1	46.5	20.7	13.4
Volatile Solid of Fermentation Medium, %	8.6%	7.9%	6.5%	3.6%
Volatile Fatty Acid in Fermentor, [mg/L]	186	155	135	110
Degradation rate, $(VS_{input}-VS_{output})/VS_{input}$	72.2%	74.5%	79.2%	88.3%
Biodiversity of Total Cells (0-1)	0.60	0.60	0.59	0.58
Biodiversity of Methanogens (0-1)	0.65	0.64	0.60	0.58
Quantitative Microscopic Fingerprinting Index (QMFI, 0-1)	0.953	0.944	0.882	0.806

To figure 53 as well as figure 54, 55, 56 for simulation: Quantitative microscopic fingerprinting index depending on operational conditions. (Number) indicates volatile solids content of fermentor (VS %).

Biogas plant	A Geesthact	B Parkstetten	C RM60 (HAW)	D Bützberg, F1	E Seth, storage	F Straw, F1 (HAW)	G Seth F2	H Brekendorf	I Bützberg, Perkolate	J Lanken, F1	K Biowerk
Total cell counts (N/mL)	7.36E+09	3.00E+10	1.98E+10	7.09E+09	1.88E+10	1.89E+10	3.78E+10	2.00E+10	1.07E+10	2.83E+10	4.68E+10
Methanogenic cell counts (N/mL)	2.32E+08	3.97E+08	7.11E+08	9.48E+08	8.23E+08	1.32E+09	1.80E+09	1.03E+09	1.04E+09	6.82E+09	5.17E+09
VFA (mg/L)	34	22900	544	3970	108	39	1336	783	86.2	186	566
VS (%)	1.5%	5.2%	2.0%	1.8%	6.9%	3.8%	7.2%	5.3%	0.8%	8.6%	3.2%
Degradation rate (%)	0.6	0.7	0.67	0.8	0.6	0.53	0.7	0.7	0.8	0.8	0.8
Diversity (0-1), methanogen	0.640	0.600	0.250	0.680	0.800	0.600	0.770	0.460	0.690	0.640	0.370
TMCP (1/cm)	0.9	11	4.2	5.83	4.6	5.1	9.83	8.6	7.18	29.7	62.47
Total Cells/Methanogens (-)	31	75.6	28	7.5	23	14.4	21	19.5	10.3	4.1	9
Diversity (0-1), Total Cells	0.630	0.570	0.650	0.400	0.500	0.530	0.500	0.430	0.400	0.600	0.640
Methanogenic cell area (μm^2)	1.33	3.32	1.7	1.17	1.19	0.75	1.19	1.73	1.19	1.07	1.61
Methanogenic autofluo-intensity	30%	83%	35%	53%	47%	52%	46%	49%	58%	41%	75%
QMFI	0.089	0.177	0.253	0.33	0.477	0.554	0.664	0.667	0.721	0.925	0.928
Substrate	Sewage sludge	Maize + Grass	Fodder beet	Biowaste	Manure + Maize	Straw	Manure + Maize	Maize	Biowaste	Maize	Biowaste
Temperature	Mesophilic	Mesophilic	Thermophilic	Mesophilic	Mesophilic	Mesophilic	Thermophilic	Thermophilic	Mesophilic	Mesophilic	Mesophilic
Type of fermentation	Wet	Wet	Wet	Dry (box)	Wet	Wet	Wet	Wet	Dry (box)	Wet	Wet

7.4. Publication activities during PhD-research

Poster, oral presentations, and project reports

Kim Y. S., Scherer P., 2013, *Rapid quantification and classification of bacteria and methanogens by digital image analysis*, VAAM Annual Conference, Bremen, Germany, **Oral presentation**, Springer Biospektrum ISSN 0947-0867, p. 55

Kim Y. S., Scherer P., 2013, *Development of a Quick Tool for Quantification of Methanogens*, Graduate School, Hamburg, Germany, **Poster presentation**

Kim Y.S., Off S., Polster E., Unbehauen M., 2013, *Establishment of core microorganisms for biogas plants – genome sequencing of isolates from biogas plants for mapping metagenome data: part 4 – methanogenic archaea*, Fachagentur Nachwachsende Rostoffe e.V. FNR 22006912, Bielefeld, **1st Oral presentation (project)**

Kim Y. S., Scherer P., 2013, *Digital Image Analysis for Quantification of Microorganisms in Environmental Habitats*. Annual conference, University of West Scotland, Paisley, Scotland, **Poster presentation**

Kim Y.S., Off S., Polster E., Unbehauen M., 2013, *Establishment of core microorganisms for biogas plants – genome sequencing of isolates from biogas plants for mapping metagenome data: part 4 – methanogenic archaea*, Fachagentur Nachwachsende Rostoffe e.V. FNR 22006912, **1st Project report**

Kim Y.S., Neumann L., Scherer P., 2013, *Einsatz einer quantitativen Bildanalyse zur Beurteilung mikrobieller Prozessparameter von Biogasanlagen*. 7th Bioenergy Forum, Rostock, Germany, **Oral presentation**, ISBN 978-3-86009-207-1, pp.339-349

Kim Y.S., Scherer P., 2013, *Digital image analysis of total cells and methanogens in anaerobic digesters to serve as fingerprint and indicator*. 13th Anaerobic Digestion Conference, Santiago de Compostela in Spain, **Poser presentation**

Kim Y. S., Scherer P., 2013, *Application of an image analysis system for quantification and classification of microbes from agricultural biogas plants*. Microscopy Conference (MC) 2013, Regensburg, Germany, **Poster presentation**

Kim Y. S., Scherer P., 2013, *Two-dimensional digital image analysis of fermentor samples for quantitative three-dimensional determination of living microorganisms,*

Promotion and Research day at the Hamburg University of Applied Sciences, Hamburg, Germany, **Poster presentation**

Kim Y.S., Goldenthal M., 2014, *Establishment of core microorganisms for biogas plants – genome sequencing of isolates from biogas plants for mapping metagenome data: part 4 – methanogenic archaea*, Fachagentur Nachwachsende Rostoffe e.V. FNR 22006912, Mainz, **2nd Oral presentation (project)**

Kim Y. S., Scherer P., 2014, *New Method to Isolate Methane Producing Bacteria under Oxygen Free Conditions,* Graduate School, Hamburg, Germany, **Poster presentation**

Kim Y.S., Goldenthal M., 2014, *Establishment of core microorganisms for biogas plants – genome sequencing of isolates from biogas plants for mapping metagenome data: part 4 – methanogenic archaea*, Fachagentur Nachwachsende Rostoffe e.V. FNR 22006912, **2nd Project report**

Kim Y. S., Scherer P., 2014, *Novel Type of a Pressurised Anaerobic Cultivation System to Isolate Hydrogenotrophic Methanogens Utilizing H2-CO2 as Single Energy and Carbon Source*, Annual conference, University of West Scotland, Paisley, Scotland, **Poster presentation**

Kim Y. S., Scherer P., 2014, *Microscopic Digital Image Analysis of a Farm-Scale Thermophilic Biogas Plant for Early Detection of Ammonia Inhibition Effects*. Biogas in Progress III, Hohenheim, Germany, **Oral Presentation,** ISBN: 978-3-940706-07-2, pp. 46-47

Kim Y. S., Scherer P., 2014, *Development and application of rapid methods for quantification and cultivation of methanogens in Bio-Methane producing fermenters*, Promotion and Research day at the Hamburg University of Applied Sciences, Hamburg, Germany. **Poster presentation**

Kim Y. S., Scherer P., 2014, *Estimation of the Total Methanogenic Cell Power (TMCP) and quantitative microscopic fingerprinting (QMF): A novel microscopic approach to forecast rapidly the performance of biogas plants*, Biogas Science 2014, Vienna, Austria, **Poster presentation**

Kim Y. S., Scherer P., 2014, *Biogas status in Germany and microscopic quantification technique*, Seminar, Seoul National University, Seoul, South Korea, **Oral presentation**

Kim Y. S., Scherer P., 2015, *Microscopic digital image analysis for monitoring of anaerobic digesters: quantification and classification of methanogens*, Seminar, Newcastle University, Newcastle, England, **Oral presentation**

Kim Y.S., Rogala M., Off S., Goldenthal M., 2015, *Establishment of core microorganisms for biogas plants – genome sequencing of isolates from biogas plants for mapping metagenome data: part 4 – methanogenic archaea*, Fachagentur Nachwachsende Rostoffe e.V. FNR 22006912, Hamburg, **3rd Oral presentation (project)**

Kim Y.S., Rogala M., Off S., Goldenthal M., 2015, *Establishment of core microorganisms for biogas plants – genome sequencing of isolates from biogas plants for mapping metagenome data: part 4 – methanogenic archaea*, Fachagentur Nachwachsende Rostoffe e.V. FNR 22006912, **3rd Project report**

Kim Y.S., Scherer P., 2015, *A New Digital Image Analysis Method for Objectification of Microbial Activity (Focused on Anaerobic Digesters)*, 12th Hamburger Studententagung zur Innovation Medizin- und Biotechnologie, Hamburg, **Oral presentation**

Kim Y.S., Steven K., Scherer P., 2015, *Digital Image Analysis as a Tool to Investigate Microbial Biodiversity*, Annual conference, University of the West of Scotland, Paisley, Scotland, **Oral presentation**

Kim Y.S., Wegner K., Off S., Scherer P., 2015, *Establishment of core microorganisms for biogas plants – genome sequencing of isolates from biogas plants for mapping metagenome data: part 4 – methanogenic archaea*, Fachagentur Nachwachsende Rostoffe e.V. FNR 22006912, **Final project report**

Journal paper publications

Kim Y. S., Westerholm M., Scherer P., 2014, *Dual investigation of methanogenic processes by quantitative PCR and quantitative microscopic fingerprinting (QMF)*, FEMS Microbiol. Lett., **360**, 76-84

Kim Y.S., and Scherer P., 2015, *Estimation of the methanogenic activity in anaerobic digesters by a new quantitative microscopic fingerprinting index (QMFI),* in preparation (95% of writing is finished)

Kim Y.S., and Scherer P., 2015, *Microscopic Digital Image Analysis of a Farm-Scale Thermophilic Biogas Plant for Early Detection of Ammonia Inhibition Effects*, in preparation (95% of writing is finished)

Kim Y.S., and Scherer P., 2015, *Automated quantification of living microbes in environmental samples using digital image analysis and a motorized epi-fluorescence microscope,* in preparation (95% of writing is finished)

Kim Y.S., Munk B., Lebuhn M., Scherer P., *Dual analysis on anaerobic digestion by mcrA gene based indicator (metabolic quotient) and quantitative microscopic fingerprinting index (QMFI),* in preparation (80% of writing is finished)

Maus I., Kim Y.S., Schlüter A., Scherer P., *Comparison of metagenomics and microscopic analysis on actual state of microbial community in four agricultural biogas plants*, in preparation (70% of writing is finished)

Cibis K., Erhard M., Hahnke S., Kim Y.S., Klocke M., Koeck D., Langer T., Maus I., Off S., Scherer P., Schlüter A., Schwarz W., 2015, *Shedding light into the black box: Comprehensive polyphasic characterization of the microbial consortium in a thermophilic full-scale biogas plant*, in preparation (90% of writing is finished)