Propionate-Oxidizing bacteria in Anaerobic Biowaste Digesters

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Abstract

The concentrations of volatile fatty acids are one of the most important parameter in anaerobic digestion of organic material e.g. in biogas plants. Thus accumulation of acetic acid, propionic acid and n-butyric acid are indicators for disturbances of the fermentation process. Especially propionic acid, as the intermediary product, whose degradation is only possible in a narrow thermodynamic window, can be found in many biogas plants. In the past many papers focused only on the different methanogenic taxa and/or did cover only a part of the known propionate-oxidizing bacteria (POB). There are even less data on thermophilic degradation of complex organic material, and an important thermophilic POB organism, *Desulfotomaculum thermobenzoicum thermosyntrophicum* has not yet been identified in situ.Thus using fluorescence in situ hybridization (FISH) not only a new FISH probe for *Desulfotomaculum thermobenzoicum thermosyntrophicum* was designed, but also already known FISH probes were used to cover all known POB species and to gain a holistic insight into propionate metabolism in biowaste digesters at different temperatures for the first time.

The main part of the actual work shows the number and species distribution of POB during mesophilic wet anaerobic digestion. The focus was especially on reactor conditions, at which an accumulation of propionic acid occured, like during re-start after revision or during overfeeding of biowaste reactors. Results of FISH investigations indicated, that the obligate syntrophic species of the genus *Pelotomaculum* sp. played the major role in propionic acid metabolism. To a lesser content *Syntrophobacter* sp. contribute to propionate degradation, whereas *Smithella propionica* was hardly present above the detection limit of 1.6×10^6 cells per mL most of the time. Within the first days of re-starting the bioreactor only the number of *Bacteria* and the concentrations of acetate and propionate increased. Not till then numbers of *Archaea* heightened, whereas the concentrations of volatile fatty acids decreased. The majority of methanogenic *Archaea* in the undigested biowaste belonged to the order of *Methanomicrobiales* and only later during the re-start process the proportion of *Methanosaeta* sp. increased. In the reactors, which were overfed with wheat-, and rye bread suspension, it could be seen, that in the recovery phase after acidification a strong decrease of biodiversity took place. Also the high sensitivity of *Methanosaeta* sp. towards ammonia concentrations > 1g per L could be demonstrated. In another experiment the numbers of POB were enriched by co-fermentation of organic waste and propionic acid.The proportion of POB to *Bacteria* could be increased to more than 20 % by propionate addition. Numbers of *Archaea* increased in parallel, mainly due to growth of *Methanomicrobiales*, whereas numbers of *Bacteria* did not vary significantly.

During dry anaerobic digestion also high numbers of *Pelotomaculum* sp. were found. The proportion of *Pelotomaculum* sp. was, however, much lower than 50 % in some assays. In these cases a majority of *Syntrophobacter* sp. could be found. As the probe Dtsyn1130 was

developed and evaluated successfully, high numbers of De*sulfotomaculum thermobenzoicum thermosyntrophicum* cells could be found in the thermophilic assays. This indicated, that this sulfate reducing species had a high relevance for the degradation of propionic acid in thermophilic biowaste digesters.

For the evaluation of probe Dtsyn1130 FISH experiments were conducted with different microorganisms (*Desulfotomaculum thermobenzoicum thermosyntrophicum*, *Desulfotomaculum thermobenzoicum thermobenzoicum*, *Desulfotomaculum thermocisternum*, *Pelotomaculum propionicicum*, *Pelotomaculum schinkii*, *Syntrophobacter fumaroxidans*, *Syntrophobacter sulfatieducens*) using different formamide concentrations. At a formamide concentration of 20 % there is already a relevant loss of fluorescence intensity in *Desulfotomaculum thermobenzoicum thermobenzoicum*, but not in *Desulfotomaculum thermobenzoicum thermosyntrophicum*.

Experiments with a propionate-degrading enrichment culture showed, that a maximum degradation of 0.97 mM propionate per d was possible. In an originally 3 organism containing culture, which uses propionate and sulfate for growth, it was possible to reduce the number of organisms by dilution and therefore results in a propionate degrading organism and a vibrio-shaped contaminant. The propionate degrader shows the highest similarity to *Syntrophobacter sulfatireducens*, if the reaction to several FISH probes and the quick propionate degradation via sulfate reduction is considered.

Zusammenfassung

Niedrige Konzentrationen flüchtiger Fettsäuren gehören zu den wichtigsten Parametern für die Beurteilung der anaeroben Vergärung von organischem Material in Biogasanlagen. Akkumulationen von Essigsäure, Propionsäure oder n- Buttersäure sind Indikatoren für Störungen der Methanogenese im Vergärungsprozess. Besonders Propionsäure als das Intermediat, dessen Abbau nur in einem engen thermodynamischen Fenster erfolgen kann, lässt sich in vielen Biogasanlagen nachweisen. Viele Arbeiten konzentrierten sich in der Vergangenheit nur auf die verschiedenen methanogenen Taxa und/oder erfassten nur einen Teil alle bisher bekannten Propionat-oxidierenden Bakterien (POB). Für den thermophilen Abbau komplexen organischen Materials ist die Datenlage noch schlechter, auch weil ein wichtiger thermophiler Organismus, *Desulfotomaculum thermobenzoicum thermosyntrophicum* bis jetzt nicht in situ identifiziert werden konnte. Aus diesen Gründen wurde unter Verwendung der Fluoreszenz in situ Hybridization (FISH) nicht nur eine neue FISH-Sonde für *Desulfotomaculum thermobenzoicum thermosyntrophicum* entworfen, sondern auch alle bereits bekannte FISH-Sonden kombiniert um erstmals alle bis jetzt bekannten POB-Spezies zu erfassen und um ein umfassendes Bild des Propionatmetabolismus zu gewinnen.

Der Hauptteil der aktuellen Arbeit befasst sich mit der Anzahl und der Artenverteilung der POB während der mesophilen Nassvergärung von Biomüllsuspensionen. Es wurden dabei speziell Bedingungen untersucht, die mit einer Akkumulation von Propionsäure assoziert werden, wie z.B. das Anfahren eines Biomüllreaktors nach Revision oder die Wiederherstellung der Methanogenese nach Überfütterung eines Bioreaktors. Die aktuelle Arbeit zeigt, dass die obligat syntrophen Spezies der Gattung *Pelotomaculum* sp. die Hauptrolle beim mesophilen Propionsäuremetabolismus spielte. In einem geringeren Maße sind beim mesophilen Propionatabbau auch *Syntrophobacter* sp. beteiligt, während *Smithella propionica* die meiste Zeit nur knapp über dem Detektionslimit von 1.6×10^6 Zellen pro mL erfasst werden konnte. Beim Anfahren des Biomüllreaktors stieg in den ersten Tagen nur die Zahl der *Bacteria* und die Konzentration von Acetat und Propionat. Erst danach stieg auch die Zahl der *Archaea* deutlich an, während die Konzentration flüchtiger Fettsäuren zurückging. Die Mehrheit der methanogenen *Archaea* im unverdauten Biomüll gehört zur Ordnung der hydrogenotrophen *Methanomicrobiales*, im Laufe des Anfahren des Bioreaktors stieg der Anteil an *Methanosaeta* sp. an. Bei den Reaktoren, welche mit Biomüll- und Weizen- oder Roggenbrotsuspensionen überlastet (überfüttert) wurden, zeigte sich in der Erholungsphase nach der Versäuerung eine starke Reduktion der Biodiversität von methanogenen und Propionat-oxidierenden Mikroorganismen. Außerdem konnte die hohe Sensitivität von *Methanosaeta* sp. gegenüber Ammoniumkonzentrationen > 1 g per L gezeigt werden. In einem weiteren Ansatz wurde durch eine Co-Vergärung von Biomüllsuspensionen mit Propionsäure die Zahl der POB gesteigert. Der Anteil von POB an der Gesamtzahl der *Bacteria* ließ sich durch Propionsäure-Zugabe auf über 20 % steigern. Parallel dazu stieg die

Anzahl der *Archaea* (hauptsächlich durch das Wachstum von *Methanomicrobiales*), während sich die Zahl der *Bacteria* kaum veränderte.

Auch bei den Untersuchungen der POB bei der Trockenvergärung von Biomüll wurde eine hohe Zahl an *Pelotomaculum* sp. entdeckt. Jedoch war der Anteil an *Pelotomaculum* sp. in einigen Ansätzen deutlich kleiner als 50 %. In diesen Fällen bildeten *Syntrophobacter* sp. im mesophilen Temperaturbereich die Mehrheit der POB. Durch die erfolgreiche Entwicklung und Evaluation der Sonde Dtsyn1130 konnte eine hohe Anzahl an Des*ulfotomaculum thermobenzoicum thermosyntrophicum* Zellen in den thermophilen Ansätzen nachgewiesen werden, was auf eine hohe Relevanz dieser Spezies von thermophilen sulfat-reduzierenden Bakterien für den Propionsäureabbau bei hohen Temperaturen hindeutet.

Für die Evaluation der Sonde Dtsyn1130 wurden FISH Experimente mit verschiedenen Mikroorganismen (*Desulfotomaculum thermobenzoicum thermosyntrophicum*, *Desulfotomaculum thermobenzoicum thermobenzoicum*, *Desulfotomaculum thermocisternum*, *Pelotomaculum propionicicum*, *Pelotomaculum schinkii*, *Syntrophobacter fumaroxidans*, *Syntrophobacter sulfatieducens*) bei unterschiedlichen Formamid-Konzentrationen durchgeführt. Bei einer Formamid-Konzentration von 20 % gibt es einen deutlichen Verlust an Fluoreszenz Intensität bei *Desulfotomaculum thermobenzoicum thermobenzoicum*, jedoch noch nicht bei *Desulfotomaculum thermobenzoicum thermosyntrophicum*.

Experimente mit einer Propionat abbauenden Anreicherungskultur zeigte, dass ein Maximum von 0.97 mM Propionat per d abgebaut werden kann. Bei einer ursprünglich aus 3 Organismen bestehenden Kultur, welche Propionat und Sulfat zum Wachstum benötigt, konnte ein Organismus durch Verdünnung entfernt werden, wodurch ein Propionat-abbauender Organismus und ein vibrio-förmiger Kontaminant übrig blieb. Der Propionat Abbauer in der Kultur ähnelt am stärksten *Syntrophobacter sulfatireducens*, wenn man die Reaktion auf verschiedene FISH Sonden und den schnellen Propionatabbau bei Sulfatreduktion berücksichtigt.

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1. Introduction

1.1 Anaerobic digestion: Development, digesters and substrates

Treatment of organic waste via anaerobic digestion (AD) has the major benefit that it produces energy in the form of biogas. Biogas from biowaste digestion is a mixture of carbon dioxide (usually less than 50 %) and methane (usually more than 50 %) and may contain trace contaminations of ammonia, hydrogen, hydrogen sulfide or other volatile compounds, depending on the substrates that are digested or on prevalent conditions for the digestion process, such as substrate pre-storage, co-substrates, organic loading rates or inhibiting compounds . Although the physiological processes were not understood in detail, it was known for centuries, that organic material that was stored or was forming sediments in an anaerobic environment generated a combustible gas. In the 19th century scientists began to elucidate the responsible mechanisms. John Dalton and Humphrey Davy, for instance, found out that the gas consisted partly of methane (Tietjen 1975). In 1868 Bechamps discovered that the gas production was due to microorganisms (McCarthy 1982). The first acidogenic organisms were isolated by Omleanski. Omleanski and Sohngen (McCarthy 1982) hypothesized that carbon dioxide and acetate were metabolized to methane by microorganisms. At the end of the 19th century the first anaerobic reactor for waste water treatment was presented (McCarthy 1982). Since the discoveries of natural gas and crude oil were numerous in the 20th century, biomethane production could not compete with fossil fuels as energy sources. As prices for natural gas and crude oil increased drastically after the year of 2000 alternative fuels and energy sources became more relevant. Intensive research of alternative sources of energy and (highly subsidized) process development for universal practical application became very attractive to satisfy the enormous energy demand of modern industrial societies and to maintain the standard of civilization. In Europe and especially in Germany a large number of more than 7000 biogas plants were installed in 20 years with an electric power output of more than 3500 megawatts (Figure 1).

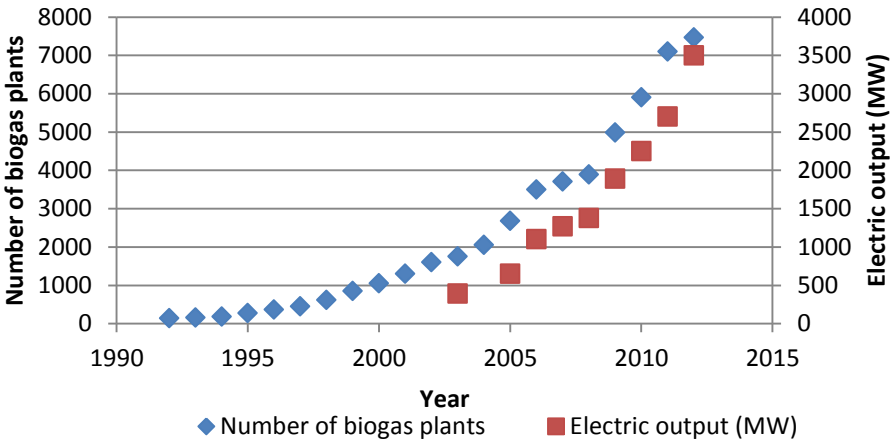

Figure 1: Number of biogas plants and their electric output in Germany. Taken from Stolpp 2010.

There are many possible organic substrate sources for biomethane production, e.g. sewage sludge, cattle, swine or chicken manure, energy crops, food left-over or municipal biowaste (e.g. the source-sorted organic fractions of municipal solid wastes). Aside of animal manures sewage sludge was a major feed stock used in anaerobic digestion (Weiland 2010). Due to subsidies from the European Union energy crops such as maize silage became attractive feed stocks, as their biomethane yield was much higher than that of sewage sludge or manure (Weiland 2010) and sales prizes of electricity were guaranteed by the renewable energy law.

There are different types of AD, which are distinguished by the temperature range or humidity of substrates for digestion. Mesophilic AD is usually carried out at temperatures between 30 – 40°C and thermophilic AD at 50 – 55°C. De Baere (2006) recorded numbers and types of biogas plants in Europe and stated, that in 2006 65 % of all digesters were operated in the mesophilic temperature range and 35 % in the thermophilic temperature range. At temperatures > 50°C there was a better hygienization of biowaste, as most of the pathogenic microorganisms, parasites, weed seeds etc. were inactivated during the hydraulic retention time in ADs at this temperature (Weiland 2010). For economic operation there were also installations for psychrophilic AD, but that technology is not widely used (O'Reilly et al. 2009).

The dry matter content of the feed substrates for AD is also an important process criterium. AD in digesters that are fed with substrates that contain more than 20 – 30 % dry matter content is defined as dry anaerobic digestion (DAD). Substrates that contain a dry matter contents of < 15 % are digested by wet anaerobic digestion (WAD). Most DAD research was done with substrates that contained 20 – 25 % dry matter (Cecchi et al. 1991, Mata-Alvarez et al. 1993, Pavan et al. 2000, Bolzonella et al. 2006), whereas for substrates that contained more than 30 % DM many authors reported a declining performance (Abbassi-Guendouz et al. 2012, Li et al. 2014a). Thermophilic AD is often combined with dry anaerobic digestion (DAD). Thermophilic DAD showed a better performance and process stability, and due to the lower water content heating costs were lower compared to WAD (de Baere 2006). For Europe de Baere (2006) reported a proportion of 44 % WAD and 56 % DAD in 2006.

1.2 Microbiology of AD under different conditions during start-up, steady state and overload

Anaerobic digestion is a complex physiological process carried out by a syntrophic interaction of prokaryotes of the domains *Bacteria* and *Archaea*. It can be divided in 4 major stages namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2). During hydrolysis complex polymeric biomolecules like carbohydrates, proteins and lipids are degraded to sugars, amino acids and fatty acids by heterotrophic anaerobic microorganisms (Li et al. 2012, Demirel and Scherer 2008). In the acidogenic stage the products of hydrolysis are further degraded to ethanol, lactate, n-butyrate, propionate, acetate, formate, CO_2 and H_2. We know from experiments with two stage AD, where the first phases of hydrolysis and

acidogenesis and the acetogenic as well as the methanogenic stage are carried out in different reactors, that different species of the genera *Aeriscardovia, Lactobacillus*, *Bifidobacteria* and others seem to play a role during the acidogenic phase (Shin et al. 2010, Xu et al. 2014). In the phase of acetogenesis all long-chain fatty acids are metabolized to acetate CO_2 and hydrogen. Many of the organisms, which are responsible for this step, are sulfate reducing bacteria (SRB) or closely related to them (Muyzer and Stams 2008). Acetogens deliver the feed for the two types of methanogens (Figure 2). Acetoclastic methanogens degrade acetate, whereas hydrogenotrophic methanogens use CO_2 and hydrogen to form methane (Table 1). Acetogenesis and methanogenesis are bond to each other in an obligately syntrophic relationship. The physiological reactions are only possible, if hydrogen is consumed by methanogens (Stams and Plugge 2009). In a perfect steady state the reactor content has a neutral pH, no detectable VFA and constant methane and CO_2 amounts. The abiotic reactor properties have their counterpart in the microbial world. In many fermenters with a perfect steady state (a high production of methane, neutral pH and low VFA) high numbers of acetate-utilizing *Methanosaeta* species were found (McMahon et al. 2004, Yu et al. 2005, Zheng and Raskin 2000, Karakashev et al. 2005), whereas high numbers of *Methanosarcina* sp., *Methanobacteriales* and *Methanomicrobiales* were detected at higher VFA concentrations (Shin et al. 2010, Blume et al. 2010). There are, especially in the order of *Methanobacteriales*, species, which can be metabolically active even at pH <6 (Blume et al. 2010).

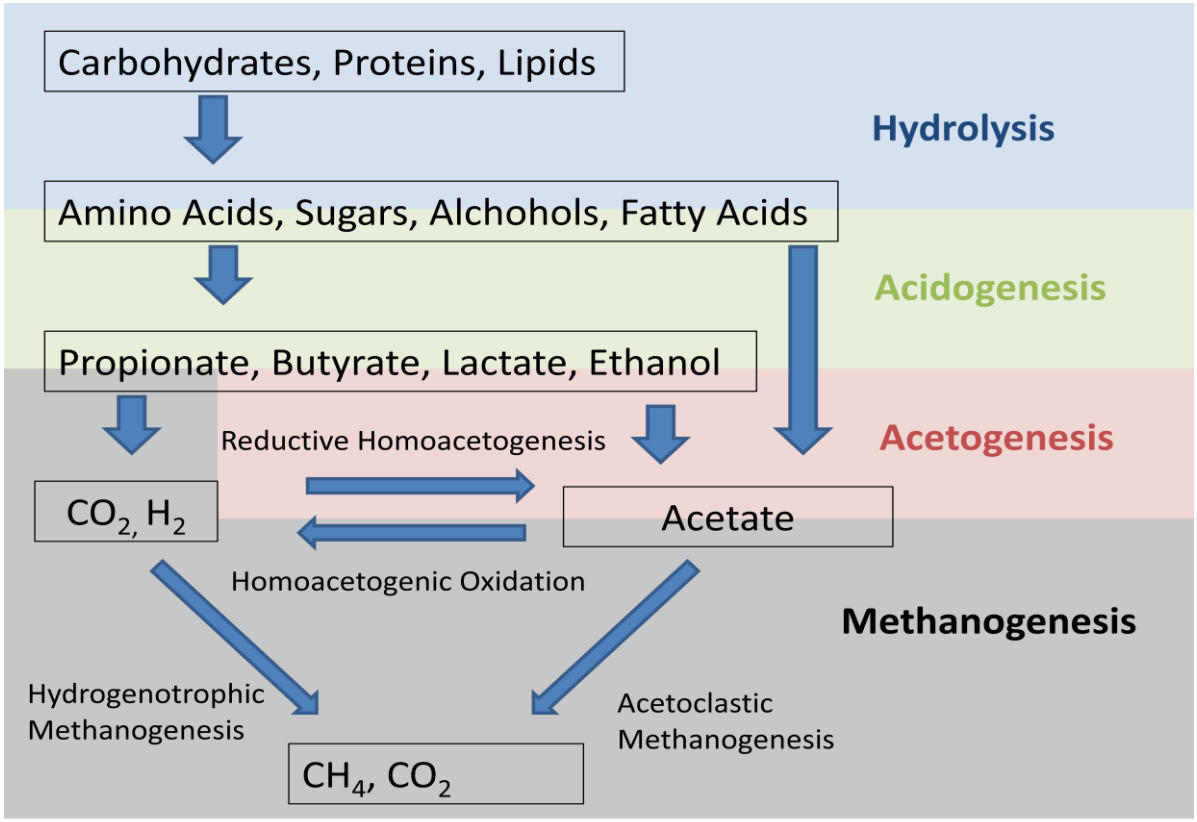

Figure 2: Simplified scheme of degradation pathways during anaerobic digestion (Demirel and Scherer 2008, Muyzer and Stams 2008).

Besides this optimal AD at steady state conditions praxis showed, that the final stage, the production of methane, carbon dioxide and the preceding acetogenic reactions linked to it by an obligate hydrogen syntrophy, is very sensitive and may fail. There are some critical situations, e.g. start-up or overload, where intermediate products, especially volatile fatty acids, accumulate. This accumulations lead to imbalances, concerning the pH and the hydrogen partial pressure, making several physiological reactions thermodynamically unfavorable. Finally methanogenesis fails, if no counteractions are undertaken (Gallert and Winter 2008).

In the first days of a reactor start-up procedure usually no or only very little methane is produced by microorganisms (Gallert et al. 2003, Gallert and Winter 2005). At this step it is important not to add to much substrate too quickly. If too much substrate is added, fast-growing acidogenic bacteria will produce high concentrations of organic acids, hence the pH will decrease and the hydrogen partial pressure will increase. Degradation of organic acids such as propionate and n-butyrate can no longer occur for thermodynamic reasons (Fig. 3). So in biowaste reactors biowaste addition has to be limited, then acetogens and methanogens can play their role in AD to metabolize hydrogen, CO_2 and volatile fatty acids finally to methane (e.g. Gallert et al. 2003, Gallert and Winter 2005).

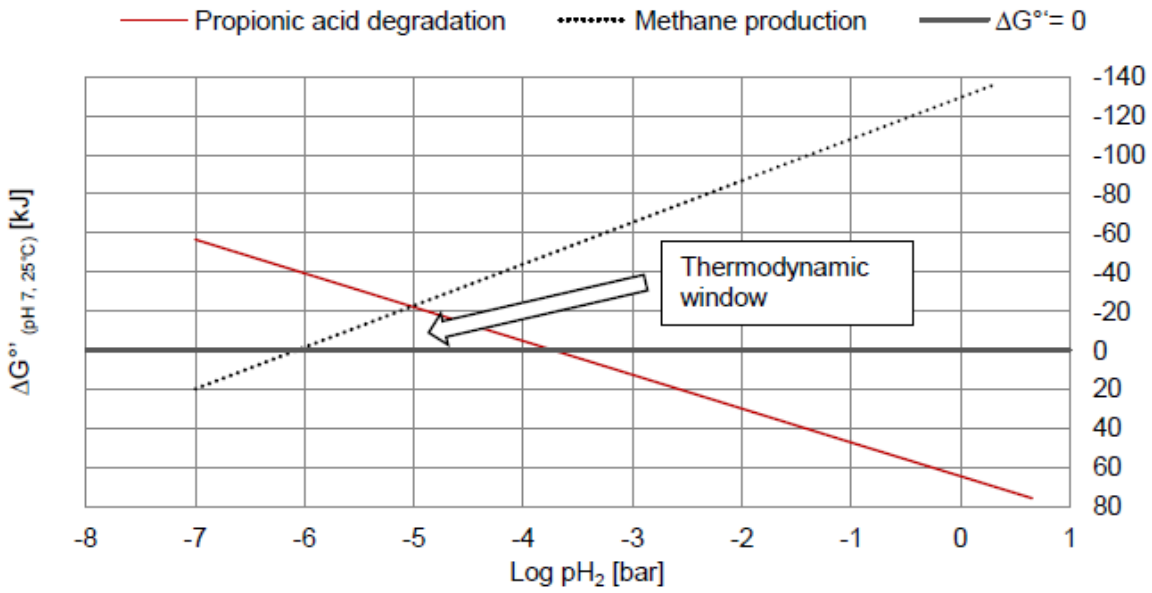

Figure 3: Thermodynamic window for methanogenic degradation of propionate (Felchner-Zwirello 2013).

There is no way to avoid completely propionic acid accumulation during start-up of biowaste digesters only by restricting the substrate supply (Gallert and Winter 2008). Some factors such as inoculum quality, organic loading rate, temperature and used substrates have a big influence (McMahon et al. 2004, Shin et al. 2010, O'Reilly et al. 2009, Griffin et al. 1998). Other influencing factors would be interspecies distances, the hydrogen partial pressure and

acetate concentrations (Felchner-Zwirello et al. 2012, 2013). The “quality” of a start-up could also be seen in the abundance of certain methanogens. McMahon et al. (2004) reported, that during start-up of continuously mixed reactors at a high loading rate or without the use of inocula numbers of *Methanosaeta* sp. decreased, whereas number of *Methanobacteriales* began to increase. In the reactor, where an inoculum was supplied and the organic loading rate was lower, it was a complete different situation: *Methanobacteriales* remained low and *Methanosaeta* numbers increased. A similar situation was reported for a mesophilic reactor by Griffin et al. (1998). Whereas propionate concentrations remained stable on a high level all the time, there was a high acetate peak and pH drop within the first days of operation and an inoculum addition was necessary to overcome the situation. At this time *Methanosaeta* sp. were washed out almost completely and a community of *Methanosarcina* and to a less extent *Methanobacteriales* took over after the pH recovery. A different situation occurred in the start-up of AD as reported by Shin et al. (2010). Acetate and to a lower extent propionate accumulated on a low level (< 0.8 g per L) within the first 10 days of start-up. *Methanosarcinales* (*Methanosaeta* and/or *Methanosarcina*), however, were washed out almost completely and *Methanomicrobiales* comprised up to 90 % of all *Archaea.* Concerning POB it was examined by McMahon et al. (2004), that *Smithella propionica* did not play a role during start-up, whereas *Syntrophobacter* remained stable or in the case *S. fumaroxidans* reacted positively to the higher offer of substrate. McMahon et al. (2004), however, did not search for *Pelotomaculum* sp.

Table 1: Standard free reaction enthalpies of fatty acid oxidation and methane production. Values calculated from the standard free formation enthalpies of the reactants at a concentration of 1 M, pH 7.0 and T = 25°C according to Thauer et al. (1977)

Reaction	G^{o2}(kJ per reaction)
Propionate- + 2 H_2O ⇒Acetate- + CO_2 + 3 H_2	+76.0
Propionate- + 2 H_2O + 2 CO_2 ⇒Acetate- + 3 $HCOO^-$ + 3 H^+	+65.3
n-Butyrate$^-$ + 2 H2O ⇒2 Acetate$^-$ + H^+ + 2 H_2	+48.3
n-Butyrate$^-$ + 2 H_2O + 2 CO_2 ⇒2 Acetate- + 2 $HCOO^-$ + 2 H^+	+38.5
4 H_2 + CO2 ⇒CH^4 + 2 H_2O	-131.7
4 $HCOO^-$ + 4 H^+ ⇒CH_4 + 3 CO_2 + 2 H_2O	-144.5
CH_3COO^- + H^+ ⇒CH_4 + CO_2	-36

The organic loading, is, however, not only important during start-up, because there is also the possibility, that a reactor, which was already started successfully, crashes and stops methanogenesis, when the OLR is raised to a too high level. There is however good reason to try the maximum OLR possible for the microbial community, as feed and biogas volume are proportional (Lindorfer et al. 2009). At which OLR a reactor suffers from overload is different, and depends on e.g. feed stock. McMahon et al. (2004) operated their digesters

successfully with an OLR of 9.4 kg VS m^{-3} day^{-1}, approaching overload conditions at 18.8 kg VS m^{-3} day^{-1}. Blume et al. (2010) fed reactors with maize silage and observed optimum conditions at 4.1 g DOM L^{-1} d^{-1} and acidification at 7.5 g DOM L^{-1} d^{-1}.

The reactions of the microbial community are similar to those reactions caused by an inappropriate or wrong start-up procedure. Thus some authors (e.g. McMahon et al. 2004, Conklin et al. 2006) observed a decline of *Archaea* at high-load conditions in anaerobic digesters. McMahon et al. (2004) saw also a decline in numbers of *Archaea.* Also the number of bacteria of the subgroups of *Methanosarcina* sp. and *Methanosaeta* sp. decreased. In the case of POB it was reported that *Smithella* sp. decreased strongly, whereas several *Syntrophobacter* species remained stable at an overload OLR of 18.8 kg VS m^{-3} d^{-1} (McMahon et al. 2004). Blume et al. (2010), who reported high numbers of *Methanosaeta* sp. at an OLR < 4 g DOM L^{-1} d^{-1}, raised the OLR further, which led to an enrichment of *Methanobacteriales*, while *Methanosaeta* sp. vanished.

1.3 Propionate oxidation and propionate-oxidizing bacteria in anaerobic digestion

There are 10 species of bacteria described, which are able to degrade propionate syntrophically (Table 2). Microscopic images of them are shown in Figure 4 (*Pelotomaculum schinkii* and *Pelotomaculum propionicicum*), Figure 5 (*Syntrophobacter fumaroxidans* and *Syntrophobacter sulfatireducens*) and Figure 6 (*Desulfotomaculum thermobenzoicum thermosyntrophicum* and *Desulfotomaculum thermocisternum*), respectively. Theories of syntrophic propionate degradation were already formulated by Bryant et al. (1967). The first tri-culture containing *Syntrophobacter wolinii*, a sulfate reducing *Desulfovibrio* and a hydrogenotrophic methanogen was described by Boone and Bryant in 1980, where *Syntrophobacter wolinii* acted as propionate degrading bacterium. Wallrabenstein et al. (1994) were able to isolate *Syntrophobacter wolinii* in pure culture and could grow it with propionate and sulfate as an electron acceptor. A year later the isolation of another species, *Syntrophobacter pfennigii* was published (Wallrabenstein et al. 1995).

Table 2: Morphology and taxonomy of syntrophic propionate-oxidizing bacteria.

Species	Morphology	Taxonomic order	Motility	Spores	Reference
Syntrophobacter wolinii	Rods, 0.6 ×1 µm, single cells pairs, chains and filaments	*Deltaproteobacteria*	-	-	Boone and Bryant 1980 Wallrabenstein et al. 1994, Liu et al. 1999
Syntrophobacter pfennigii	Egg shaped rods, 1–1.2 × 2.2–3µm, single, pairs, chains	*Deltaproteobacteria*	-	-	Wallrabenstein et al. 1995
Syntrophobacter fumaroxidans	Rods eye shaped, 1.1–1.6 × 1.6–2.5 µm single, pairs	*Deltaproteobacteria*	-	-	Harmsen et al. 1998
Syntrophobacter sulfatireducens	1–1.3 × 1.8–2.2 µm, single, pairs, chains	*Deltaproteobacteria*	-	-	Chen et al. 2005
Smithella propionica	Rods, 0.5 × 3–5 µm, some 10 µm	*Deltaproteobacteria*	~	-	Liu et al. 1999
Pelotomaculum schinkii	Rods, 1 × 2–2.5 µm	*Firmicutes*	-	+	de Bok et al. 2005
Pelotomaculum propionicicum	Rods, 1 × 2–4 µm, single cells, pairs	*Firmicutes*	-	+	Imachi et al. 2007
Pelotomaculum thermopropionicum	Rods, 0.7–08 × 1.7–2.8 µm, single cells	*Firmicutes*	-	+	Imachi et al. 2002
Desulfotomaculum thermobenzoicum thermosyntrophicum	Rods with pointed ends, 1 × 3–11 µm	*Firmicutes*	~	+	Plugge et al. 2002
Desulfotomaculum thermocisternum	Straight rods, 0.7–1 × 2–5.2 µm, flagella	*Firmicutes*	+	+	Nilsen et al. 1996

After 1995 more propionate-oxidizing organisms (POB) were described. In 1999 researchers reported a new propionate degrading genus, *Smithella,* with *Smithella propionica* as a type strain (Liu et al. 1999). Liu et al. (1999) did also experiments to determine the temperature and pH range and the maximum specific growth rate for *Syntrophobacter wolinii* in methanogenic associations. Nilsen et al. (1996) found the first thermophilic POB originating from a Norwegian oil reservoir formation water. Then *Desulfotomaculum thermobenzoicum thermosyntrophicum* was described by Plugge et al. (2002). In the same year the first

member of the genus *Pelotomaculum* was described (Imachi et al. 2002).The later on discovered *Pelotomaculum schinkii* and *Pelotomaculum propionicicum* were the first obligately syntrophic POB, hence no axenic cultures exist.

Table 3: Physiology of syntrophic propionate-oxidizing bacteria. References for the single species see Table 2.

Species	pH	Temperature	Specific growth rate per d (axenic/ syntrophic)	Axenic substrate use	Syntrophic substrate use
Syntrophobacter wolinii	6.1–7.4	28–37°C	0.062/0.022–0.3	Pyruvate, Fumarate, Malate, Propionate + SO_4	Propionate
Syntrophobacter pfennigii	6.2–8	20–37°C	0.07/0.066	Lactate + SO_4, Propionate + SO_4	Lactate, Propionate, Propanol
Syntrophobacter fumaroxidans	6.2–8	20–40°C	0.024/0.17	Fumarate, Malate, Aspartate, Pyruvate, Propionate + Fumarate, H_2+Fumarate, Formate + Fumarate Propionate+ SO_4 H_2 CO_2+SO_4 Succinate+ SO_4 Formate+ SO_4	Propionate
Syntrophobacter sulfatireducens	6.8–8.8	20–48°C	0.12/very low	Pyruvate + SO_4 Propionate +SO_4	Propionate
Smithella propionica	6.4–7.4	28–37°C	very low/0.29	Crotonate	Propionate
Pelotomaculum schinkii	7	37°C	ND/0.59	None	Propionate, Propionate + Fumarate
Pelotomaculum propionicicum	6.5–7.5	25–45°C	ND/0.2	None	Propionate
Pelotomaculum thermopropionicum	6.7–7.5	45–65°C	1.6/ 0.19	Pyruvate, Fumarate	Propionate, Lactate, Ethanol, Ethylene glycol, 1-Propanol, 1-Butanol, 1-Pentanol, 1,3-Propanediol
Desulfotomaculum thermobenzoicum thermosyntrophicum	6–8	45–62°C	0.099/0.115	Propionate +SO_4, Lactate +SO_4, Pyruvate+ SO_4, H_2 CO_2+SO_4, Benzoate, Glycin, Pyruvate, Fumarate, Lactate	Propionate
Desulfotomaculum thermocisternum	6.2–8.9	41–75°C	ND/ND	H_2 CO_2, Propionate, Lactate, Pyruvate, n-Butyrate, C5-C10, C14–C17 Ethanol, Propanol, Butanol	Propionate

Although POB species are scattered in taxonomically distant orders of Gram negative *Deltaproteobacteria* on the one hand and Gram positive *Firmicutes* on the other hand, they have one feature in common, namely their ability to use sulfate as an electron acceptor, or in the case of the obligate syntrophic POB, their close relatedness to sulfate-reducing bacteria (Müller et al. 2010, McInerney et al. 2008). It was hypothesized, that their syntrophic relationship to methanogenic bacteria was due to sulfate limitations in some habitats. If that is true, then *Pelotomaculum schinkii* and *Pelotomaculum propionicicum* (Figure 4) are the latest step of evolution in sulfate-limited environment, as these organisms seem to have lost their ability for sulfate reduction. Findings of Imachi et al. 2006 confirm this hypothesis.

All yet known POB are rod shaped bacteria, and diameters differ slightly between 0.5 (*Smithella propionica*) to 1.6 µm (*Syntrophobacter fumaroxidans*, see Figure 5). There is however a difference concerning length of single bacteria between *Syntrophobacter wolinii* (1µm) and *Desulfotomaculum thermobenzoicum thermosyntrophicum* (11 µm, Figure 6, Table 2). Members of the genera *Pelotomaculum* and *Syntrophobacter* (with the exception of one species) did not show any motility. *Smithella propionica* and *Desulfotomaculum thermobenzoicum thermosyntrophicum* were slightly motile, whereas *Syntrophobacter pfennigii* was only motile during the exponential growth phase. Flagella were only reported for *Desulfotmaculum thermocisternum* (Table 2). Spore formation was reported for all species of the order *Firmicutes*, as well as a positive Gram reaction, whereas a negative Gram reaction and no spores could be found in all *Deltaproteobacteria* species (Table 2).

The physiological properties of POB are summarized in Table 3. Species of the genus *Syntrophobacter* and *Desulfotomaculum thermobenzoicum thermosyntrophicum* are able to reduce sulfate and use this ability to grow on propionate in pure cultures (McInerney et al. 2008). All members of the genus *Pelotomaculum* instead lack the ability to use sulfate, sulfite or organosulfonates (Imachi et al. 2006). Besides the syntrophic or sulfate reducing propionate degradation many organisms can ferment intermediary products of the methylmalonyl degradation pathway, such as pyruvate and fumarate. Also lactate can serve as a carbon source for many POB (Table 3). The obligately syntrophic POB and *Smithella propionica* can be considered as the specialists, if we take into account their narrow substrate spectrum. On the other hand we find generalists such as *Syntrophobacter fumaroxidans*, *Pelotomaculum thermopropionicum* and *Desulfotomaculum thermocisternum*. Concerning *Desulfotomaculum thermocisternum* it should be mentioned, that neither Imachi et al. (2000) nor Plugge et al. (2002) nor in the actual work *Desulfotomaculum thermocisternum* could be grown on propionate and sulfate or on propionate with a syntrophic methanogenic partner. Indeed no report could be found besides the original paper of Nilsen et al. (1996). All POB could be described as neutrophilic, though some have a broader pH tolerance spectrum than others. *Pelotomaculum thermopropionicum, Pelotomaculum propionicicum* and *Smithella propionica* are the most sensitive organisms. The highest tolerance towards alcalinic pH was reported for *Syntrophobacter sulfatireducens* (pH 8.8) and the highest acid tolerance for

Desulfotomaculum thermobenzoicum thermosyntrophicum (pH 6) (Table 3). Concerning temperature we clearly see difference between the thermophilic *Firmicutes* within *Desulfotomaculum* or *Pelotomaculum thermopropionicum* and the mesophilic *Firmicutes* and *Deltaproteobacteria*. Except for the species where we do not know their temperature range (e.g. *Pelotomaculum schinkii*), it can be seen that some trend to be more tolerant to low or high temperatures than others. *Pelotomaculum propionicicum* and *Syntophobacter sulfatireducens* (Figure 5) for instance, grow even at 45 and 48°C. On the other hand the temperature range of *Smithella propionica* is limited (Table 3). The species description of Boone and Bryant (1980) did not contain a temperature and pH range, but later papers quoted, that *Syntrophobacter wolinii* had the same narrow temperature range (Liu et al. 1999). A special aspect, which is due to syntrophic growth of POB, is that consortia and granules are formed with the syntrophic partner to avoid a high interspecies distance (Stams and Plugge 2009, Felchner-Zwirello et al. 2013). If *Pelotomaculum thermopropionicum* was grown syntrophically on propionate it formed dense granules, whereas after growth on ethanol no aggregation could be observed (Ishii et al. 2005). Felchner Zwirello et al. (2013) reported that distances lower than 0.29 µm are strongly beneficial for growth.

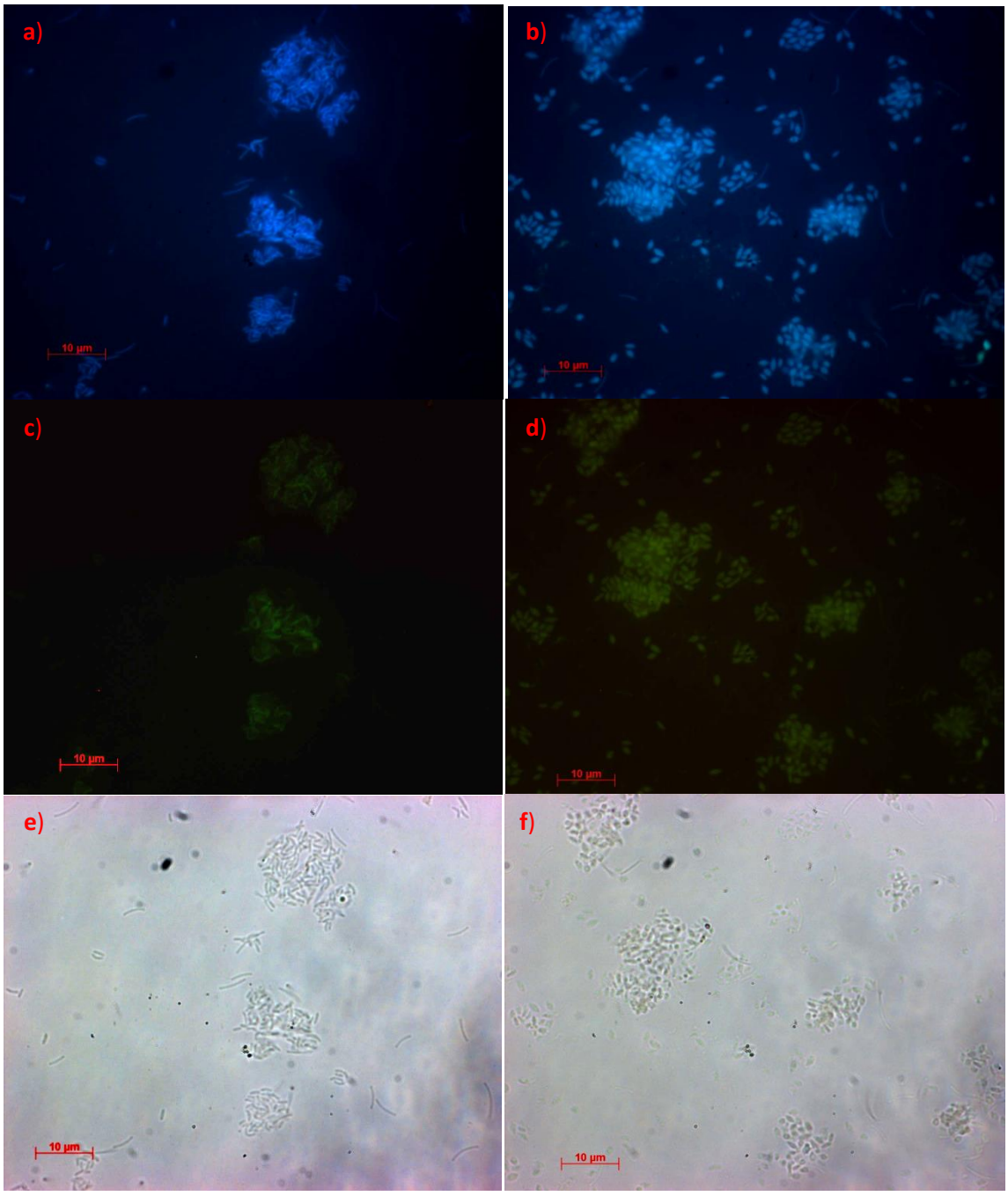

Figure 4: Images of *Pelotomaculum propionicicum* (a, c, e) and *Pelotomaculum schinkii* (b, d, f) after DAPI staining, FISH(a, b), using Eub388 probe with FAM fluorescence (c, d) and by phase contrast microscopy (e, f).

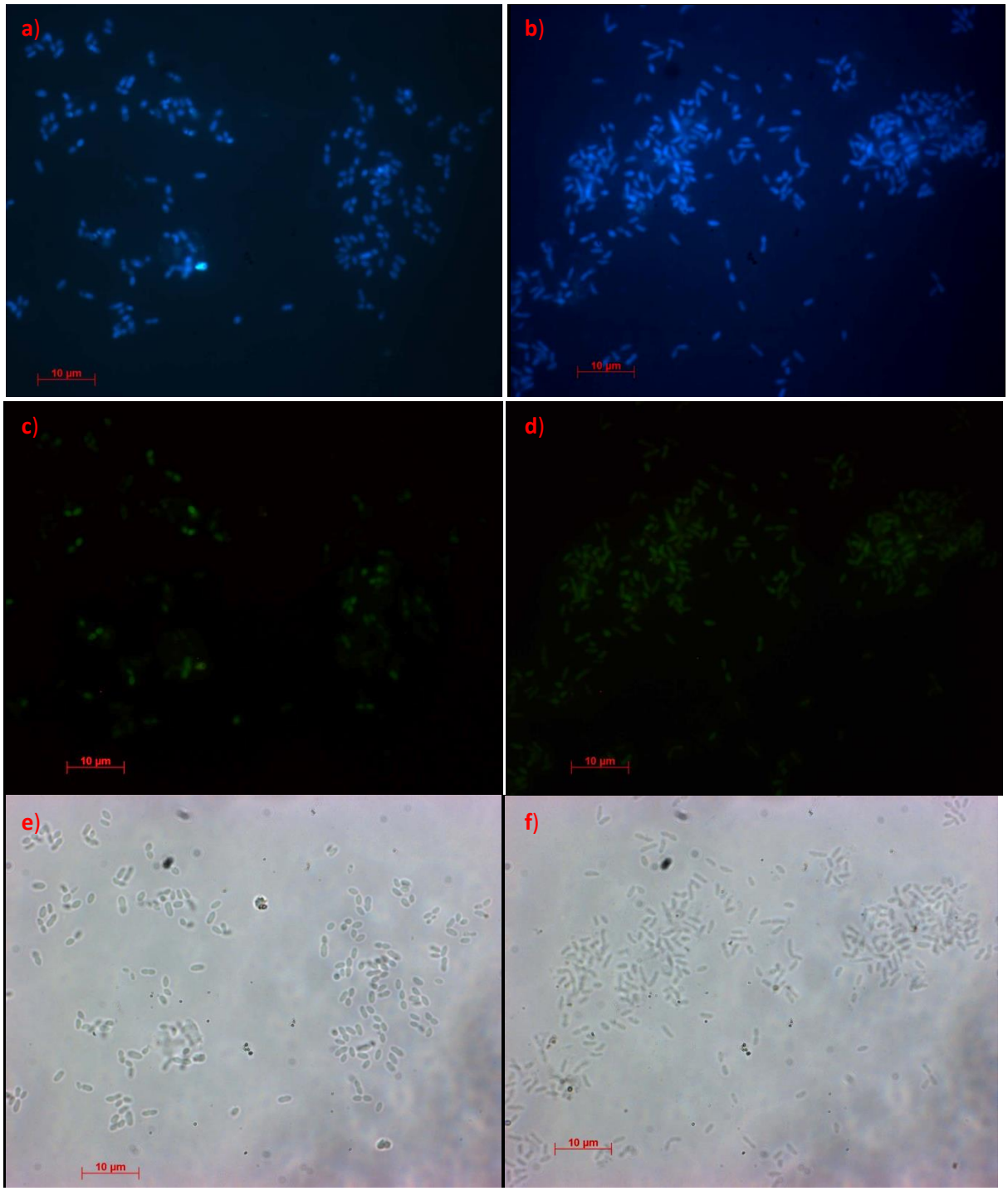

Figure 5: Images of *Syntrophobacter fumaroxidans* (a, c, e) and *Syntrophobacter sulfatireducens* (b, d, f) after DAPI staining (a, b), FISH using Eub388 probe with FAM fluorescence (c, d) and by phase contrast microscopy (e, f).

Another important feature is the specific growth rate of POB. In Table 3 the growth rates for pure cultures and for syntrophic bi-cultures of POB were compiled. In general the genera *Pelotomaculum* and *Smithella* tend to have higher specific growth rates than *Syntrophobacter* and *Desulfotomaculum*. Interestingly pure cultures of *Syntrophobacter sulfatireducens* and *Syntrophobacter pfennigii* grown on propionate and sulfate have higher growth rates as compared to growth rates obtained in syntrophic associations. For

Syntrophobacter wolinii Wallrabenstein et al. (1994) reported a similar relation (0.062 vs 0.022) between pure cultures grown on propionate and sulfate and methanogenic syntrophic bi-cultures. Liu et al. (1999) reported that the highest possible growth rate of *Syntrophobacter wolinii* was 0.3 per d. A similar relationship was found for *Syntrophobacter fumaroxidans*. The highest specific growth rate of POB was reported for *Pelotomaculum schinkii*. It has to be mentioned however, that the very high growth rate of 0.59 (generation time of 1.5 d) was achieved by excessive pre-enrichment of the methanogenic partner before the inoculation of the POB *Pelotomaculum schinkii* (de Bok et al. 2005).

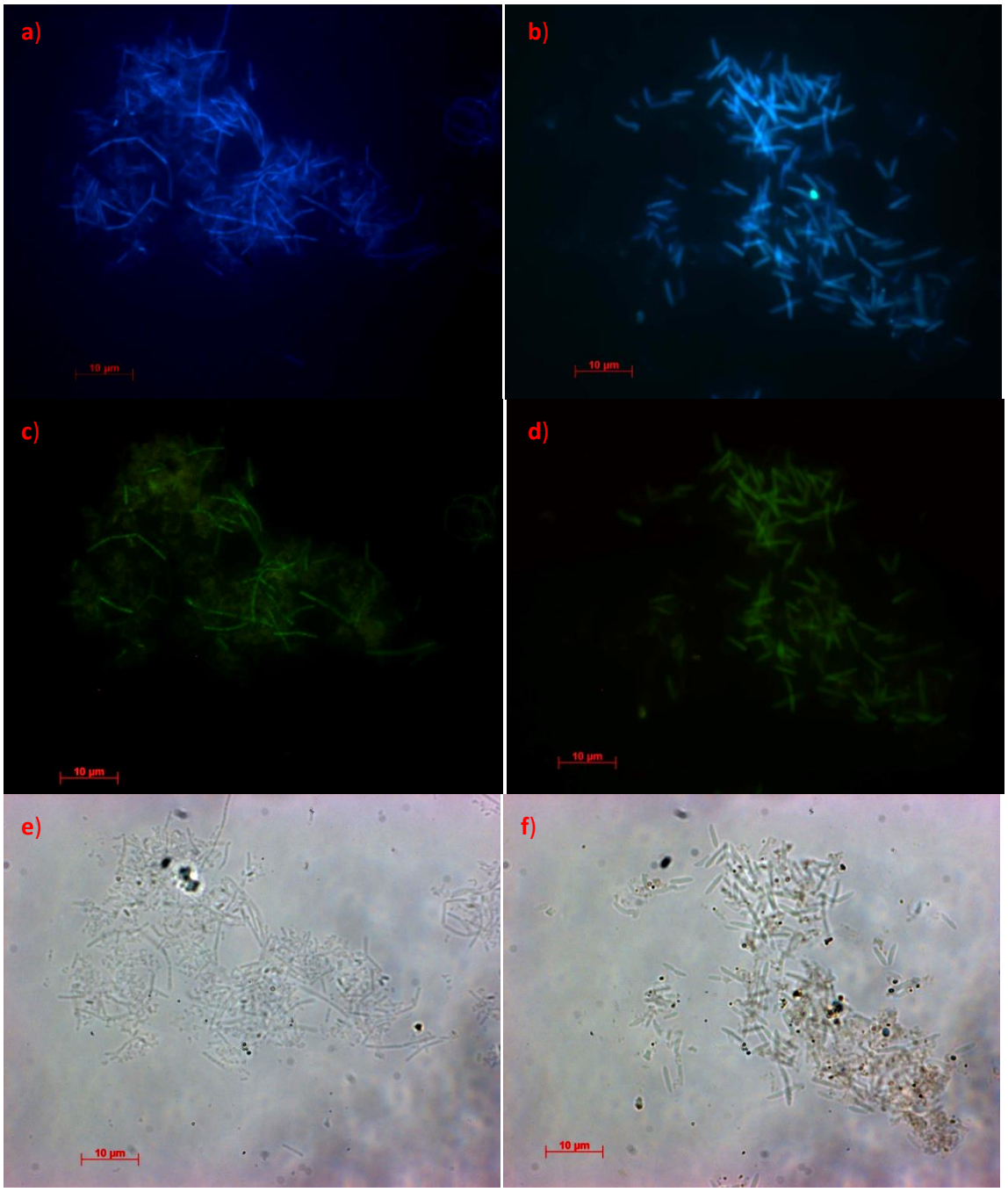

Figure 6: Images of *Desulfotomaculum thermocisternum* (a, c, e) and *Desulfotomaculum thermobenzoicum thermosyntrophicum* (b,d,f) after DAPI staining (a, b), FISH using Eub388 probe with FAM fluorescence (c, d) and by phase contrast microscopy (e,f).

Fermentative growth is however not superior in every species. For *Smithella propionica* growth on crotonate is quite unfavourable (Liu et al. 1999) and *Syntrophobacter fumaroxidans* showed a higher growth rate, when grown on fumarate (0.09) than when grown on propionate with sulfate (0.024) as an electron acceptor. Syntrophic propionate degradation achieved even better results (0.17) (Harmsen et al. 1998). *Desulfotomaculum thermobenzoicum thermosyntrophicum* grows almost equally well on propionate with sulfate as an electron acceptor as on propionate in syntrophic association with methanogens. Unfortunately there were no data published for growth rates of *Desulfotomaculum thermocisternum* in the paper of Nilsen et al. (1996).

There are two metabolic pathways, how propionate can be degraded by syntrophic POB. The first is the degradation via methylmalonyl CoA and succinate. It was already known from organisms, which can degrade propionate with the help of sulfate reduction (but cannot degrade it in syntrophic association), that degradation goes via methylmalony CoA and succinate (Kremer and Hansen 1988). Houwen et al. (1990) found out that the same pathway is used by a methanogenic syntrophic co-cultures of *Syntrophobacter wolinii*. Thus from 1 mol propionate (+ 2 mol H_2O) 1 mol acetate, 1mol CO_2 and 3 mol H_2 were produced (Table 1, Figure 7). In fact most authors concluded that hydrogen diffuses in the form of formate (de Bok et al. 2002, Dong et al. 1994, Boone et al. 1989). De Bok et al. (2003) reported two tungstate-containing formate dehydrogenases, which have very high formate oxidation and CO_2 reduction rates. Plugge et al. (2009) found out, that methanogenic co-cultures of *Syntrophobacter fumaroxidans* and *Methanospirillium hungatei* grown in media without tungsten and molybdenum showed only poor growth, whereas pure cultures of *Syntrophobacter fumaroxidans* grown on fumarate were not affected by tungsten depletion.

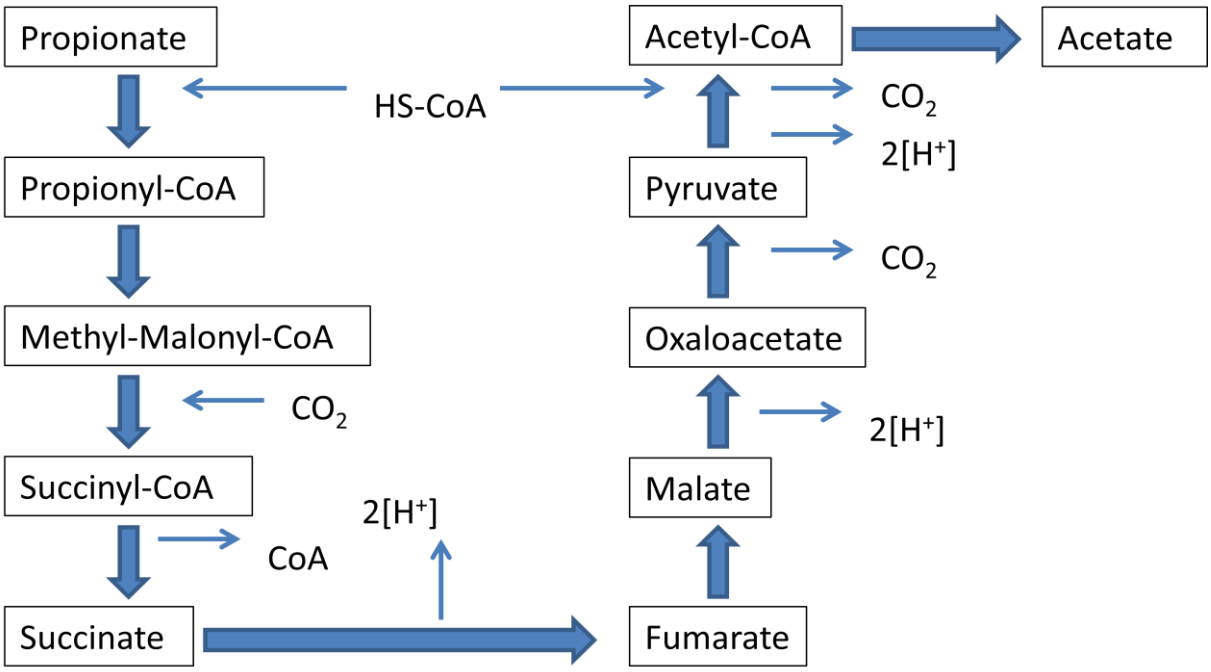

Figure 7: Scheme of Propionate degradation via the methylmalonyl-CoA pathway (Plugge et al. 1993).

The degradation of propionate via the methylmalonyl-CoA pathway was also reported for the organism later described as *Syntrophobacter fumaroxidans* (Plugge et al. 1993). The pathway was elucidated by using C^{13}/C^{14} isotope labelled carbon and enzyme activity tests, where strong activities of acetate HS-CoA transferase and propionate HS-CoA transferase were found (Plugge et al. 1993). For the other pathway, performed only by *Smithella propionica,* little information is available in literature. De Bok et al. (2001) proposed this pathway as a result of experiments with ^{13}C-isotope-labeled propionate. The main difference is that 2 propionate molecules condense to form 3-keto,2-methylpentanoate, which after successive reactions finally dismutate and form n-butyrate and acetate. The n-butyrate is then degraded to 2 acetate molecules (Figure 8).

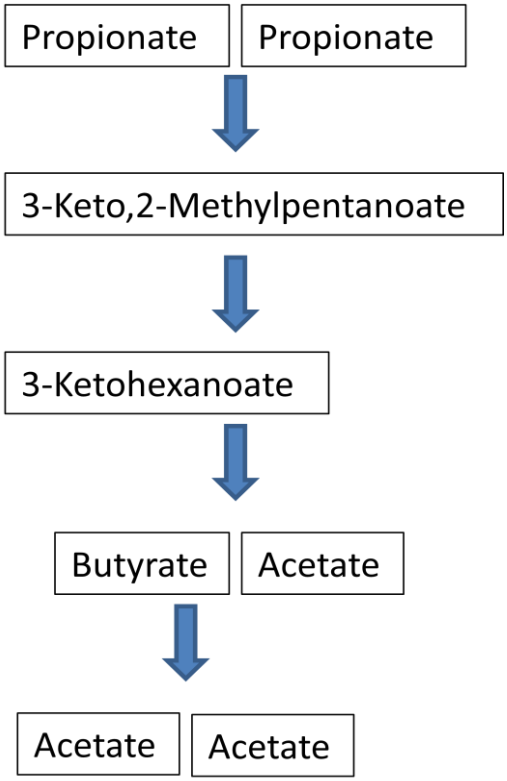

Figure 8: Scheme of propionate degradation via the C6-dismutation pathway (de Bok et al. 2001).

Besides the 16s rRNA gene sequences we have the complete genome sequence of *Syntrophobacter fumaroxidans* and *Pelotomaculum thermopropionicum*, which also confirmed the prior described degradation physiology (Plugge et al. 2012, Kosaka et al. 2008). In a co-culture of *Syntrophobacter fumaroxidans* the transcription levels of genes for formate dehydrogenases and hydrogenases were examined using qPCR. It was revealed, that substrate and the methanogenic partner (*Methanospirillum hungatei* or *Methanobacterium formicicum*) have an effect on which dehydrogenases and hydrogenases are transcribed (Worm et al. 2011). Gene expression under different growth conditions of *Pelotomaculum thermopropionicum* was examined with the help of a microarray (Kato et al. 2009), results were similar to the findings of Worm et al. (2011). In another approach Imachi et al. 2006

searched for the gene dsrAB, a key gene for sulfate reduction. The gene was found in *Pelotomaculum propionicicum*, but not in the co-cultures of *Pelotomaculum schinkii* and *Pelotomaculum thermopropionicum*, hence it was concluded, that adaption to syntrophic lifestyle was an evolutionary current event.

1.4 Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) is a valuable technique of molecular biology to characterize microbial communities in situ and has proven its effectiveness many times for selective quantification of microorganisms (Li et al.2011, Karakashev et al. 2005, Zahedi et al. 2013) or to show the in-situ distribution of different microorganisms (Hori et al. 2006). Other authors used special variations of FISH such as MAR FISH (Ariesyadi et al. 2007a) or CARD FISH (Cardinali-Rezende et al. 2012)

The principle of FISH is to insert an oligonucleotide into cells, which is specific for the searched microorganism and which can be detected by epifluorescence or confocal laser microscopy (Pernthaler et al. 2001). The first detection of a specific sequence of nucleotides in situ was reported by Gall and Pardue (1969), who used radioactive labeled oligonucleotides. Later, per se unspecific fluorescent dyes were combined with specific oligonucleotide probes. The advantage was that these probes were not radioactive and emitted a light signal only at a specific wavelength, which made it possible to detect more than one group of organisms in a sample. For in situ identification of phylogenetic groups the best way is to use oligonucleotides, which match with sequences of the 16s rRNA, as a huge database of sequences exist (Pernthaler et al. 2001). Many of presently used protocols were developed from the work of Amann et al. (1990). Like with other methods, where oligonucleotides are involved, a step, where oligonucleotides form bonds and a step where not perfectly matching pairings are washed away, were combined. To achieve this two steps the hybridization and the washing step were necessary. The washing step was carried out at slightly higher temperatures, so that all bonds of probes, which did not show a perfect match were broken and the fluorescent oligonucleotide was washed away (Pinkel 1999). Factors, as temperature, the content of NaCl and the formamide concentration are important to dissolve and bind oligonucleotides. The bonds between oligonucleotides cause a specific melting temperature, at which double stranded oligonucleotides are melted to single stranded oligonucleotides (Pinkel 1999, Pernthaler et al 2001). Most reports in which FISH was used, work with a constant temperature and influence the melting temperature by using higher or lower stringency conditions (lower or higher formamide content). If stringency is too low, then the risk to stain cells not selectively enough is given. Alternatively, if it is too high no staining is achieved (Pinkel 1999). Thus every probe had to be tested in a series of formamide concentrations, ideally with target and close-related non target organisms (e.g. containing 1 mismatch in the respective sequence) (Pernthaler et al. 2001).

The main advantage of FISH is, that cells are discreted and besides the specific fluorescence signal, information is gained about cell distribution in situ. Approaches like with PCR, qPCR methods are very sensitive to methodological errors, e.g. the extraction of nucleic acids can be a critical step and gene copies are counted, which need not to match with the number of cells, as some sequences can occur more than once in a cell (Daims 2009). There are, however, also other reasons, which can falsify results obtained with FISH. One problem, especially when working with samples originating from biowaste is the presence of many autofluorescent particles. Another problem would be the low penetration of the fluorescing nucleotides into the probe, and thus not achieving a clear fluorescence signal. Similar unreliable results are obtained, if selected organisms have a low concentration of ribosomes (Daims 2009; Pernthaler et al. 2001).

Many gene probes, which are important to examine microbial community structures during anaerobic digestion, were developed by Raskin et al. (1994). For the first time these probes made the main orders of methanogenic *Archaea* distinguishable. Although Crocetti et al. (2005) revealed some weak points and suggested improved new gene probes, in actual research the "old" gene probes are used, e.g. by Zahedi et al. (2013) or by Montero et al. (2009). The probes relevant for POB were developed by various scientists. Ariesyadi et al. (2007b) for instance developed the genus level probe synbac824, specific for *Syntrophobacter* sp. and Harmsen et al. (1995) introduced species-specific probes designed for *Syntrophobacter fumaroxidans* and *Syntrophobacter pfennigii*, and a year later a probe specific for *Syntrophobacter wolinii* (Harmsen et al. 1996). Imachi et al. (2006) was able to design a genus-level probe for the *Desulfotomaculum* subcluster Ih, which was later described as *Pelotomaculum*. Nahihiro et al. (2012) modified the probe slightly by prolonging the sequence. There is, however, no species-level probe *Pelotomaculum propionicicum* available, only for *Pelotomaculum schinkii* and for *Pelotomaculum thermopropionicum* probes exist (Narihiro et al. 2012). As there are only few studies on POB in thermophilic digesters no probes specific for *Desulfotomaculum* species relevant for syntrophic propionate oxidation exist, thus the actual work introduces a new probe specific for *Desulfotomaculum thermobenzoicum thermosynthrophicum*.

1.5 Aims of the work

As the degradation of propionic acid is one of the major metabolic processes during anaerobic digestion, it is important to know, how it is degraded, when degradation fails and when it works best. Thus it is important to understand the organisms, which actually are responsible for this process, to make anaerobic digestion more efficient and to increase the amount of methane.

In this work the main intention was to determine the number of POB, methanogens and domains in methanogenic reactors at different feeding regimes and different stages of anaerobic digestion, such as start-up, overload as well as during feeding of various co-

substrates. Most other studies on POB focused on special organisms or quantified only a selection of the known organisms as part pro toto for all POB. In this work instead all known POB species were covered, using different oligonucleotide probes and combinations of them. In the case of *Desulfotomaculum thermobenzoicum thermosyntrophicum* a new probe was designed, to have a more complete insight in the thermophilic anaerobic digestion. The second step of analysis was to find out how the data of microorganism abundances were linked to the abiotic properties of the reactors. To summarize the major goals of the thesis the following questions are formulated:

- What is the proportion between *Bacteria* and *Archaea*? What is the relationship between DAPI cell counts and the sum of the cells, which are covered by the two domain FISH probes?
- Which methanogenic order has the highest abundance? What role plays the concentrations of VFA, ammonia and pH? Is there a difference between rye and wheat bread as co-substrates? What is the effect of propionate co-feeding?
- Which POB are the dominant subgroup? Are there certain reactor conditions, which favor the occurrence of certain species?

2. Material und Methods

2.1 Media and Microorganisms

The media used for cultivation of propionate-oxidizing bacteria are listed in Table 1, followed by a detailed composition.

Table 4: Overview of different media used for experiments

Media	Name of media	Used for cultivation of	Gas phase
Medium A	Mineral Medium for propionate degraders	*Syntrophobacter sulfatireducens*, *Syntrophobacter wolinii*, Mesophilic propionate-oxidizing, Bi-culture, Mesophilic propionate-oxidizing syntrophic culture	N_2
Medium B	MPOB Medium	*Syntrophobacter fumaroxidans* *Desulfotomaculum thermobenzoicum thermosyntrophicum*	N_2/CO_2
Medium C	*Pelotomaculum* Medium	*Pelotomaculum propionicicum* *Pelotomaculum schinkii*	N_2/CO_2
Medium D	*Smithella* Medium	*Smithella propionica*	N_2/CO_2
Medium E	*Pelobacter propionicus* Medium	*Syntrophobacter pfennigii*	N_2/CO_2
Medium F	*Desulfobacter* Medium	*Desulfotomaculum thermobenzoicum thermobenzoicum*	N_2/CO_2
Medium G	*Desulfovibrio* Medium	*Desulfotomaculum thermocisternum*	N_2

The strains *Pelotomaculum propionicicum*, *Pelotomaculum schinkii*, *Smithella propionica*, *Syntrophobacter* wolinii, *Syntrophobacter pfennigii*, *Syntrophobacter fumaroxidans*, *Syntrophobacter sulfatieducens* were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen(DSMZ, Braunschweig) as living culture, the strains *Desulfotomaculum thermobenzoicum thermobenzoicum*, *Desulfotomaculum thermobenzoicum thermosyntrophicum*, *Desulfotomaculum thermocisternum* were also obtained from DSMZ as freeze dried cultures.

A propionate enrichment culture was obtained by Monica Felchner-Zwirello. The culture originated from the full scale digester plant Karlsruhe Durlach and was transferred into fresh media several times (Felchner-Zwirello 2013).

The mesophilic propionate-oxidizing culture was also obtained by Monica Felchner-Zwirello. The culture originated from an industrial-size mesophilic anaerobic reactor. The culture consisted of three different species at the beginning, when the culture was handed over. Further dilution and transfer into new medium resulted in the loss of one contaminant, thus the culture was named as mesophilic propionate-oxidizing bi-culture.

Medium A: Mineral Medium for propionate degraders

Ingredients for 1 L medium

NH_4Cl	0.50 g
K_2HPO_4	1.74 g
$CaCl_2$/$MgCl_2$ solution	1.00 mL
$NaHCO_3$	3.80 g
SL 10 solution	1.00 mL
Se-WO_4	0.50 mL
Wolfes` vitamins	1.00 mL
Resazurin (0.1 %)	0.10 mL
Reducing agent	10.00 mL

<u>Selenite-tungstate solution (Tschech and Pfennig 1984)</u>

NaOH	500 mg
Na_2SeO_3 x 5 H_2O	3 mg
Na_2WO_4 x 2 H_2O	4 mg
H_2O bidest.	1000 mL

<u>SL 10 solution (Tschech and Pfennig 1984)</u>

HCl 25 % (v/v)	10 mL
$FeCl_2$ x 4 H_2O	1500 mg
$ZnCl_2$	70 mg
$MnCl_2$ x 4 H_2O	100 mg
H_3BO_3	6 mg
$CoCl_2$ x 6 H_2O	190 mg
$CuCl_2$ x 2 H_2O	2 mg
$NiCl_2$ x 6 H_2O	4 mg
Na_2MoO_4 x 2 H_2O	36 mg
H_2O MilliQ	990 mL

<u>$CaCl_2$ $MgCl_2$ solution</u>

$CaCl_2$ x 2 H_2O	6.00 g
$MgCl_2$ x 6 H_2O	6 g
H_2O MilliQ	100 mL

<u>Reducing agent solution</u>

NaOH (1M)	10 mL
Resazurin (0.1 %)	0.1 mL
Na_2S x 9 H_2O	1200 mg
H_2O MilliQ	90 mL

<u>Wolfe's vitamin solution (10fold concentrated) (Balch et al. 1979)</u>

D (+) Biotin	20 mg
Folic acid	20mg
Pyridoxamindihydrochloride	100 mg
Thiaminiumdichloride	50 mg
Riboflavin	50 mg
Niacin	50 mg
Ca-(D+)-Pantothenate	50 mg
Cyanocobalamin	10 mg
p-Aminobenzoic acid	50 mg
DL-α-Lipoic acid	50 mg
H_2O MilliQ	1000 mL

All ingredients (except the reducing agent solution and the 3.8 g $NaHCO_3$) were mixed with 986.2 mL MilliQ water and pH adjusted at 7.4. The medium was heated in a Schott flask to

make it anaerobic. As soon as the medium boiled the Schott flask was closed with a rubber stopper, the air atmosphere was evacuated at a gassing station and replaced by N_2 gas. The procedure was repeated 2 more times to eliminate all oxygen in the medium. When the medium cooled down the reducing agent was added with a syringe. For preparation of the reducing agent NaOH, resazurin and H_2O were mixed, boiled and then Na_2S x 9 H_2O was added. The solution was filled into a serum bottle, closed with a rubber stopper and the air exchanged with N_2 gas. After adding 10 ml of reducing agent solution to 1 l of medium the medium was transferred together with the 3.8 g $NaHCO_3$ into the anaerobic chamber, where the solid $NaHCO_3$ was brought into solution in the medium. Then medium was distributed in 40 ml portions into serum bottles, which were closed with rubber stoppers. After transfer out of the anaerobic chamber another gas exchange for nitrogen was made at the gassing station before autoclaving the medium.

Medium B: MPOB Medium (Stams et al. 1993)

Ingredients for 1 L medium

Na_2HPO_4 x 2 H_2O	0.53 g
KH_2PO_4	0.41 g
NH_4Cl	0.30 g
$CaCl_2$ x 2 H_2O	0.11 g
$MgCl_2$ x 6 H_2O	0.10 g
NaCl	0.30 g
SL 10 solution (see medium A)	1.00 mL
Selenite-tungstate solution (see medium A)	1.00 mL
Yeast extract	0.20 g
Resazurin (0.1 %)	0.1 mL
$NaHCO_3$(stock solution 80 g per L)	4.00 g
Wolfe's vitamin solution (see medium A)	1.00 mL
Na_2-fumarate (1M stock solution/Na_2-Propionate(1M stock solution)	2.00 g
Na_2S x 9 H_2O (stock solution 100 g per L)	0.50 g

Preparation was similar to the preparation of medium A. Sterile anaerobic stock solutions for vitamins, $NaHCO_3$, Na_2S x 9 H_2O and the substrate (Na_2-fumarate) were prepared under N_2 atmosphere or and under N_2/CO_2 (only for the $NaCO_3$ stock solution). Medium-specific amounts were added after autoclaving. The rest of the ingredients were mixed and water was added to a total volume of 1L. The medium was heated near the boiling point and 38.3 mL distributed to serum bottles, which were sealed with rubber stoppers. The gas phase was exchanged against an N_2/CO_2 atmosphere and the medium was autoclaved. Final pH was checked and adjusted between 7.0 and 7.2, if necessary.

Medium C: *Pelotomaculum* Medium (Imachi et al. 2000)

Ingredients for 1 L medium

Na_2-Propionate	(1M stock solution)	2.00 g
KH_2PO_4		0.14 g
$MgCl_2$ x 6 H_2O		0.20 g
$CaCl_2$ x 2 H_2O		0.15 g
NH_4Cl		0.54 g
Yeast extract		0.10 g
Wolfe's vitamin solution (see medium A)		1.00 mL
SL 10 solution (see medium A)		1.00 mL
Selenite-tungstate solution (see medium A)		1.00 mL
Resazurin (0.1 %)		0.50 mg
$NaHCO_3$ (stock solution 80 g per L)		2.50 g
Cysteine-HCl x H_2O (stock solution 120 g per L)		0.30 g
Na_2S x 9 H_2O (stock solution 100 g per L)		0.30 g

Preparation was similar as for Medium B. Additionally a cysteine stock solution under N_2 atmosphere was prepared. Final pH was checked to be at 7.

Medium D: *Smithella* Medium (Liu et al. 1999)

Ingredients for 1 L medium

NH_4Cl	1.00 g
$MgCl_2$ x 6 H_2O	1.00 g
$CaCl_2$ x 2 H_2O	0.40 g
KH_2PO_4	0.40 g
Wolfe's mineral elixier	1.00 mL
Yeast extract	2.00 g
Trypticase peptone	2.00 g
Na-crotonate (1M stock solution)	1.70 g
Wolfe's vitamin solution (see medium A)	1.00 mL
Resazurin (0.1 %)	0.1 mL
$NaHCO_3$ (stock solution 80 g per L)	2.00 g
Mercaptoethanesulfonic acid (stock solution 100 g per L)	0.50 g
Na_2S x 9 H_2O (stock solution 100 g per L)	0.30 g

Wolfe's mineral elixier:

$MgSO_4$ x 7 H_2O	30.00 g
$MnSO_4$ x H_2O	5.00 g
NaCl	10.00 g
$FeSO_4$ x 7 H_2O	1.00 g
$CoCl_2$ x 6 H_2O	1.80 g
$CaCl_2$ x 2 H_2O	1.00 g
$ZnSO_4$ x 7 H_2O	1.80 g
$CuSO_4$ x 5 H_2O	0.10 g
$KAl(SO_4)2$ x 12 H_2O	0.18 g
H_3BO_3	0.10 g
Na_2MoO_4 x 2 H_2O	0.10 g
$(NH_4)2Ni(SO_4)2$ x 6 H_2O	2.80 g
Na_2WO_4 x 2 H_2O	0.10 g
Na_2SeO_4	0.10 g
H_2O MilliQ	1000.00 mL

Preparation was similar as for medium B. Additionally a mercaptoethane sulfonic acid stock solution was prepared under a N_2 atmosphere. The final pH was checked and adjusted between 7.0 and 7.2 if necessary.

Medium E: *Pelobacter propionicus* Medium (Wallrabenstein et al. 1995)

Ingredients for 1 L medium

Na_2-Propionate (1M stock solution)	
KH_2PO_4	0.20 g
NH_4Cl	0.25 g
NaCl	1.00 g
$MgCl_2$ x 6 H_2O	0.40 g
KCl	0.50 g
$CaCl_2$ x 2 H_2O	0.15 g
SL-10 solution (see medium A)	1.00 mL
Seven vitamins solution	1.00 mL
Resazurin (0.1 %)	0.10 mL
$NaHCO_3$ (stock solution 80 g per L)	2.50 g
Na_2S x 9 H_2O (stock solution 100 g per L)	0.36 g

Seven vitamins solution

Vitamin B_{12}	100.00 mg
p-Aminobenzoic acid	80.00 mg
D(+)-Biotin	20.00 mg
Nicotinic acid	200.00 mg
Calcium pantothenate	100.00 mg
Pyridoxine hydrochloride	300.00 mg
Thiamine-HCl x 2 H_2O	200.00 mg
H_2O MilliQ	1000.00 ml

Preparation was similar as for medium B. The final pH was checked to be at 7.2.

Medium F: *Desulfobacter* Medium

Ingredients for 1 L medium

Na_2SO_4	3.00 g
KH_2PO_4	0.20 g
NH_4Cl	0.30 g
NaCl	21.00 g
$MgCl_2$ x 6 H_2O	3.10 g
KCl	0.50 g
$CaCl_2$ x 2 H_2O	0.15 g
Na-L-lactate (1M stock solution)	2.50 g
$NaHCO_3$ (stock solution 80 g per L)	5.00 g
Na_2S x 9 H_2O (stock solution 100 g per L)	0.40 g
Resazurin (0.1 %)	0.1 mL
Selenite-tungstate solution (see medium A)	1.00 mL
SL 10 solution (see medium A)	1.00 mL
Wolfe's vitamin solution (see medium A)	1.00 mL

Preparation was similar as for medium B. The final pH was checked to be between 7.0 and 7.4.

Medium G: *Desulfovibrio* Medium

Ingredients for 1 L medium

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	1.0 g
$CaCl_2$ x 2 H_2O	0.1 g
$MgSO_4$ x 7 H_2O	2.0 g
Na-L-lactate (1M stock solution)	2.0 g
Yeast extract	1.0 g
Resazurin	1.0 mg
$FeSO_4$ x 7 H_2O	0.5 g
Na-thioglycolate	0.1 g
Ascorbic acid	0.1 g

Solutions of Na-thioglycolate, ascorbic acid and FeSO4×7H_2O were prepared separately as 10 mL stock solutions under a N_2 atmsophere. The rest of the ingredients were mixed with 980 mL water, boiled in a Schott bottle, sealed with a rubber stopper and cooled down under a N_2 atmosphere. Medium and stock solutions were transferred into the anaerobic chamber were all the solutions were mixed together and 40 mL portions were distributed to serum bottles. After leaving the anaerobic chamber gas was changed another time to N_2 at the gassing station and bottles were autoclaved. The final pH was checked to be at 7.8.

2.2 Sample sources

The biowaste for all experiments originated from the large scale wet anaerobic digestion plant of Karlsruhe in Karlsruhe/Durlach. As in many German cities the organic fraction of the municipal waste is collected separately with rotating drum trucks (Gallert et al. 2003). Before digestion in the full scale, completely mixed anaerobic reactor the collected biowaste is squeezed and metals are removed by an over-band magnet. The such pretreated biowaste is mixed with process water in the ratio 1:2 and de-fibered in a hydro-pulper. After or during pulping, undesired heavy material (e.g. metals, glass, gravel, porcelain pieces) is passing a grit at the bottom of the hydro-pulper and is removed with a spiral pump, whereas light plastic material is forming a scum layer and removed from top with a rake. Afterwards fine sand is removed from the suspension with the help of a hydro cyclone(Gallert et al. 2003). The reactor is a cylindrical tank reactor with gas introduction by mixing. The total volume is 1350 m^3, the working volume ranges from about 640 m^3 up to 1000 m^3. Since sand removal by the hydro-cyclones was not complete, it was sedimented at the bottom of the reactor and had to be removed every 2 – 4 years during extended maintenance periods. At the time when the reactor was re-started after maintenance, samples were collected during the first 19 days and after 81, 101 and 181 days for population analyses (Moertelmaier et al. 2014).

The properties of the biowaste suspension, originating from the hydro pulper are complied in Table 5.

Table 5: Composition of fresh and digested biowaste suspensions. (Taken from Moertelmaier et al. 2014).

Parameters	Fresh biowaste[a]	Digested biowaste[b]
Total solids, TS (%)	6.0	4.0
Volatile solids, VS (%)	4.6	2.2
Chemical oxygen demand, COD (g per L)	98	38
Total Kjeldahl nitrogen, TKN (g per L)	2.2	2.6
Ammonia (g per L)	0.5	0.7
pH	4.5	7.3
Acetate (g per L)	3.3	0
Propionate (g per L)	3.1	0
n-Butyrate (g per L)	1.5	0

[a,b] Mean of triplicate analyses (± 5 %) of 8 batches of fresh biowaste, taken over one year[a] and of digested biowaste taken as inoculum[b]. Suspensions were sieved through 0.4 x 0.4 cm grits under circular shaking.

Figure 9: Photograph of 10 L Lab scale reactors.

For the DAD experiments non-processed and non-wettened biowaste was used. Non-organic material (plastic bags, metals, etc.) were sorted out per hand and then the biowaste was shredded with a cutter. Experiments of dry anaerobic digestion were performed in 3L glass reactors. To obtain less than 30 % TS content biowaste was diluted with tap water (Li et al. 2014a).

For the WAD co-digestion experiments with bread and propionate 10 L lab scale reactors (working volume 8 L) were used. Biogas production was measured with wet gas-meters (Figure 9). The biowaste material was taken after de-fibering in the hydropulper and de-sanding by hydro-cyclones (Table 5). In the lab reactors biowaste was re-circulated with pumps from top to bottom for mixing. To maintain a temperature suitable for mesophilc prokaryotes (37°C), water from a heated water bath was pumped through silicon tubing, which were wrapped around the reactors (Li et al. 2015b). Samples (10 ml) from the lab scale reactors were taken through a sampling tube. The first 5 ml of effluent were discarded.

2.3 Volatile fatty acids gas chromatography

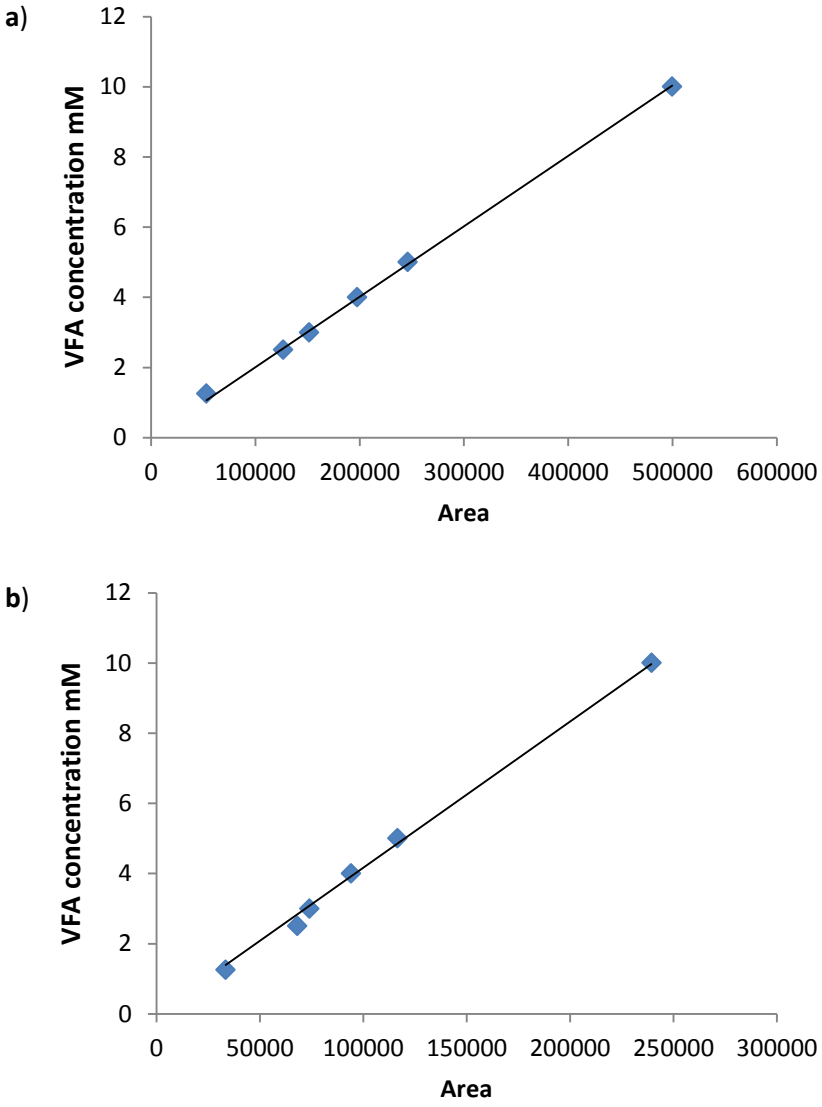

Figure 10: Propionate (a) and acetate (b) calibration with peak areas measured by GC for different defined standards.

A volume of 0.1 mL sample was centrifuged and the supernatant were diluted with 4 % phosphoric acid. Samples from cultures containing mineral media were diluted 1:1. Samples, which were withdrawn directly from biowaste reactors, were diluted 1:3. A quantum of 1 µl was injected directly with a syringe. Gas chromatography using N_2 as a carrier gas and Chromosorb C101 in a Teflon column was used as stationary phase. The injector temperature was set at 210°C, the detector temperature at 210°C and the oven temperature at 180°C. Detection was performed by a flame ionization detector with a hydrogen flame. For calibration samples with 1.25, 2.5, 3, 4, 5, 10 mM propionate and acetate were prepared and measured (Figure 10). This led to the following equation for quantification.

Propionate: $VFA\ concentration = \ 0.2 \times 10^5 \times area;\ R^2 = 0.9991$

Acetate: $VFA\ concentration = \ 0.42 \times 10^5 \times area; R^2 = 0.9961$

2.4 Ion Chromatography (IC)

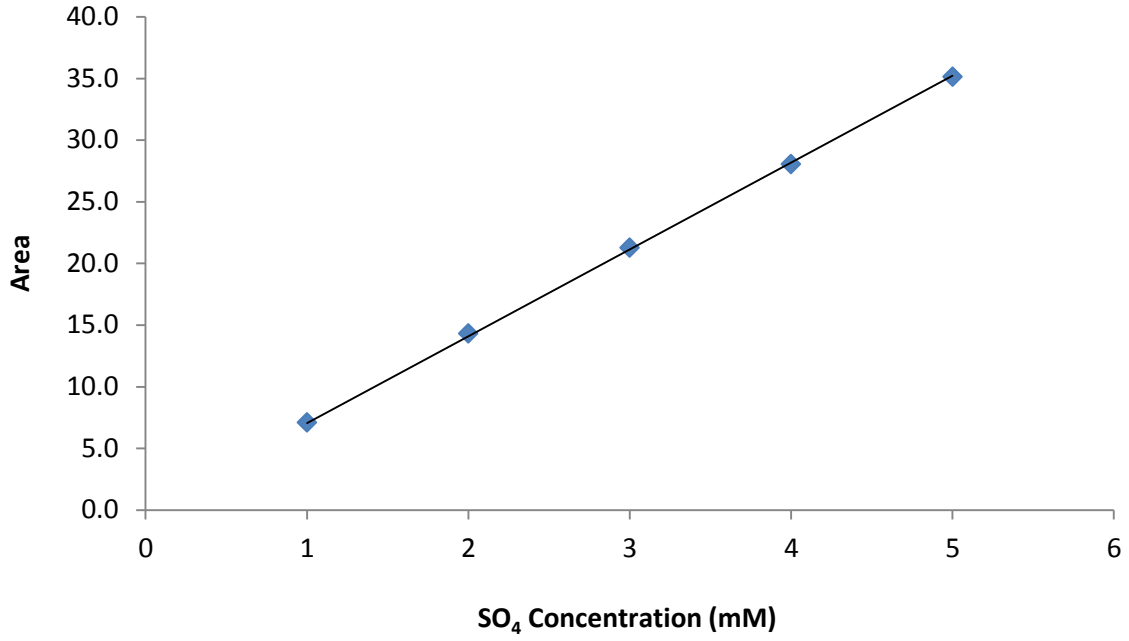

Figure 11: Sulfate calibration with the peak areas measured by IC for different defined standards.

For Ion Chromatography an ICS-90 Ion Chromatograph from Dionex was used. The Ion Chromatograph was equipped with an Ion-Pac AS9-HC column and a suppressed conductivity detector. The device is able to detect anions and cations. For the detection of anions a 90 mM Na_2CO_3 solution served as eluent (flow rate: 1 ml per min) and for regeneration of the column a regenerator solution is used namely a 36 mM sulfuric acid solution. To avoid spoilage of the device all samples were centrifuged at 15000 rpm for 5 min. Of the supernatant 200µL were pipetted in 2 ml Eppendorf reaction tubes and mixed with 1800µl Milli-Q water to have a dilution ratio of 1:10. The diluted samples were injected into the system (1 ml sample volume).

Sulfate sodium salt standard solutions were used for a calibration equation. The salt was diluted in ultra-pure Milli-Q water at concentrations 1 mM, 2 mM, 3 mM, 4 mM and 5 mM (Figure 11). Data of peak retention time and peak area were calculated with the software Chromeleon version 6.60.

$$Sulfate\ concentration = \ area \div 7.0435;\ R^2 = 0.9998$$

2.5 Fixation

Two solutions were required for fixation of bacteria. The first solution was prepared by dissolving 4 g of para-formaldehyde in 65 mL preheated water. A few drops of 2M NaOH were added until all para-formaldehyde was dissolved at slightly alkaline pH. The second solution contained a 3-fold concentrated phosphate buffered saline solution (PBS). Thirty-five mL of the second solution were mixed with 65 ml of the first solution resulting in a 4 %

(w/v) para-formaldehyde concentration. After filter sterilization the solution was stored at -20°C until use . Portions of 0.1 mL of each sample were pipetted into 2 mL reaction tubes and mixed with 0.3 ml of 4 % para-formaldehyde solution (Amann et al. 1990). The mixture was incubated at 4°C for 3 h and then centrifuged at 15000 rpm for 5 minutes in a Microfuge (Eppendorf, Hamburg, Germany). The pellet was washed in PBS. Centrifugation and washing were repeated 2 times. Finally 1 ml of a 1:1 mixture of PBS and 100 % Ethanol (HPLC-purified) was added to the pellet. Samples were frozen at - 20°C in 1 ml 50 % ethanol-PBS-solution before further analysis.

2.6 FISH

FISH experiments were performed as described previously (Amann et al. 1990, Felchner-Zwirello et al. 2013, see also Figure 12).

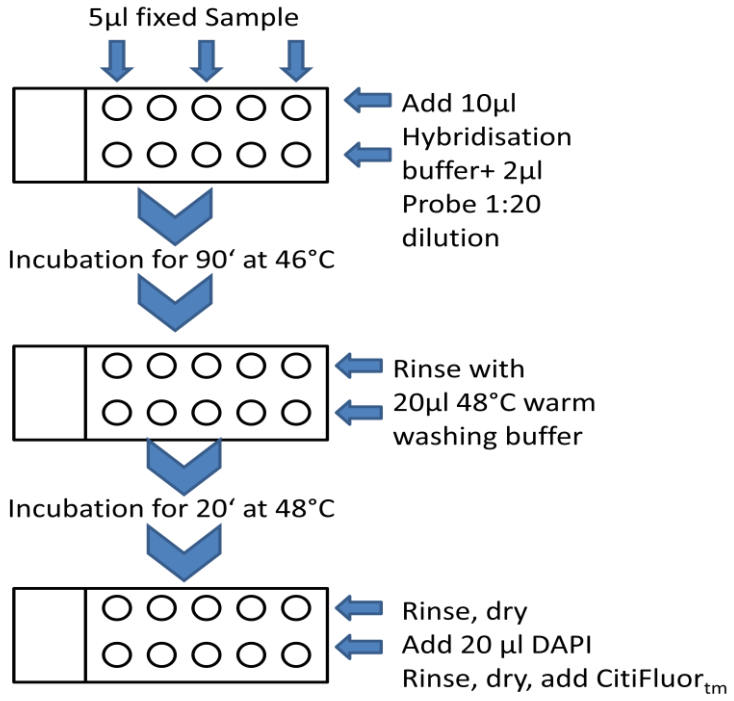

Figure 12: Sample preparation for fluorescence in-situ hybridization (FISH) prior to Microscopy

The amount of 5 µl of sample volume was pipetted on a Teflon coated slide with 8mm wells. First the samples were air dried and then completely dehydrated by an ascending ethanol concentration series, using increasing ethanol concentrations of 50, 80 and finally 99 %. The exposure time to each concentration was one minute. Hybridization buffer and the washing buffer were prepared freshly for each FISH experiment according to the specific formamide concentration needed by the used gen-probe (Table 6, Table 7). Oligonucleotide gen-probes were diluted with water. For each sample 10 µl hybridization buffer (Amann et al. 1990) and 2 µl of the diluted gen-probe solution were mixed and incubated for 90 min. at 46°C, then washed with washing buffer, which was pre-heated to 48°C (Amann et al. 1990), and

incubated for 20 min. at 48°C in a water bath. After the incubation with washing buffer samples were washed another time with water and air-dried. All samples were counter-stained with 0.1 µM 4',6-diamidino-2-phenylindol (DAPI) hence 20 µL of DAPI solution was used for each sample . CitiFluor™ was used as an embedding agent.

Table 6: Hybridization buffers for FISH.

Form amide concentration (%)	5M NaCL	TRIS buffer pH 8	Sodium dodecyl sulfate 10 % (w/v)	Form amide	MilliQ Water
0	360	40	4	0	1600
5				100	1500
10				200	1400
15				300	1300
20				400	1200
25				500	1100
30				600	1000
35				700	900
40				800	800
45				900	700
50				1000	600
55				1100	500
60				1200	400

Table 7: Washing buffer for FISH.

Formamide concentration (%)	5M NaCL	NaCl mol per L	TRIS buffer pH 8	Sodium Dodecyl Sulfate 10 % (w/v)	EDTA pH8
0	9000	0.900	1000	50	0
5	6300	0.636			
10	4500	0.450			
15	3180	0.318			
20	2150	0.225			500
25	1490	0.159			
30	1020	0.112			
35	700	0.080			
40	460	0.056			
45	300	0.040			
50	180	0.028			
55	100	0.020			
60	40	0.008			

Table 8: Oligonucleotide probes with fluorescence markers (5'-FAM[1] or 5'Cy3[2]).

Probe[1,2]	Target	Formamide (%)	Sequence 5'-3'	Reference
Eub388[1]	most *Bacteria*	30	GCT GCC TCC CGT AGG AGT	Amann et al. 1990
Arc915[2]	*Archaea*	30	GTG CTC CCC CGC CAA TTC CT	Stahl and Amann, 1991
Mg1200[1]	*Methanomicrobiales*	30	CGG ATA ATT CGG GGC ATG CTG	Raskin et al.1994
Mb310[2]	*Methanobacteriales*	30	CTT GTC TCA GGT TCC ATC TCC G	Raskin et al. 1994
MsMx860[2]	*Methanosarcinales*	30	GGC TCG CTT CAC GGC TTC CCT	Raskin et al. 1994
Mx825[1]	Genus *Methanosaeta*	30	TCG CAC CGT GGC CGA CAC CTA GC	Raskin et al. 1994
Glh821m[1]	Genus *Pelotomaculum*	10	ACCTCCTACACCTAGCACCC	Narihiro et al. 2012
Synbac824[2]	Genus *Syntrophobacter*	10	GTA CCC GCT ACA CCT AGT	Ariesyady et al. 2007b
SmiSR354[1]	*Syntrophus* group incl. *Smithella propionica*	10	CGC AAT ATT CCT CAC TGC	Ariesyady et al. 2007b
SmiLR150[2]	*Smithella* sp. long rod (LR)	10	CCT TTC GGC ACG TTA TTC	Ariesyady et al. 2007a
MPOB1	*Syntrophobacter fumaroxidans*	30	ACG CAG GCC CAT CCC CGA A	Harmsen et al. 1998
KOP1	*Syntrophobacter pfennigii*	30	TCA AGT CCC CAG TCT CTT CGA	Harmsen et al. 1998
Dtsyn1130[1]	*Desulfotomaculum thermobenzoicum thermosyntrophicum*	20	GGT TAA GTC ACA GCA ACG AGC G	Actual work

2.7 Microscopy

A Zeiss Axioskop A50 equipped with a mercury HBO 50 UV lamp and an Axiocam camera served for microscopy and photography, respectively. All microphotographs were taken at a magnitude of 1200 under immersion oil. The 16s RNA probes were labeled with either 5'-FAM or '5-Cy3. The filters specific for DAPI, 5'-FAM and '5-Cy3 are characterized in Table 9. From each sample 4 photomicrographs were taken with DAPI specific filter, with 5'-FAM specific filter, with 5'-Cy3. Images were analyzed with the help of Axiovision 3.1 or DAIME software (Daims et al. 2006). The software detects all fluorescent particles in a picture, thus it was necessary to sort out *fluorescing* particles other than the bacteria. Another problem was that the software did not count correctly, when two or more bacteria were very closely attached at each other. Image corrections were made manually by taking into account criteria such as particle size and form, color and intensity of fluorescence, and by comparing DAPI, phase contrast and FISH images of the same view field.

To get the cell numbers per mL the following calculation was done:

$$Number\ of\ cells\ per\ mL = cells\ in\ viewfield\ \times \frac{area\ of\ vial}{area\ of\ viewfield} \times \frac{1000\mu l}{volume\ of\ sample\ \mu l} \times dilution$$

The view field of the axiocam was 98µm in length and 65µm in broad. The vials of the Teflon coated slides were 8 mm in diameter. Taxa, which were not found in 10 microscopic view fields were considered not at all present or only at lower numbers than 1.58 x10^6 cells per ml.

Table 9: Zeiss filter sets used for microscopy of DAPI, FAM and Cy3 labeled bacterial samples.

Filter name	Excitation	Beam splitter	Emission	Used for Fluorescent dye
Zeiss Filter set 05	BP 395–440	LP 460	LP 470	DAPI
Zeiss Filter set 09	BP 450–490	FT 510	LP 515	FAM
Zeiss Filter set 20	BP 546/12 (HE)	FT 560 (HE)	BP 607/80 (HE)	Cy3

2.8 Probe Design and Evaluation

Probe was found by comparing the 16s rRNA gene sequences with the help of the database provided by the National Center for Biotechnology Information (NCBI) and their basic local alignment search tool (BLAST). Sequences, which showed the highest number of mismatches were tested with the oligonucleotide analyzing software Oligo Analyzer 1.0.3 by Teemu Kuulasma (2011) to fulfill the properties summarized in Table 10.

Table 10: Desired parameters for oligonucleotides to fulfil requirements for new FISH probes

Probe property	Desired probe parameters
Probe length	18–25 bases
GC content	40–60 %
T_m	>56°C
Maximum stretch of the same base	3

To proof the specificity of the probe for FISH pure cultures of the target organism and various closely related more distant related species should be tested. For the target organism *Desulfotomaculum thermobenzoicum thermosyntrophicum* FISH experiments using probe Dtsyn1130 at different formamide concentrations (10,20, 25,30, 35, 40, 45, 50, 60 % formamide, see Table 6 and Table 7) were performed. The closest related organism *Desulfotomaculum thermobenzoicum*, which belong to the same species, was equally treated and FISH experiments were undertaken for the same formamide concentrations. For *Desulfotomaculum thermocisternum*, *Pelotomaculum schinkii*, *Pelotomaculum propionicicum* and *Syntrophobacter sulfatireducens*, FISH experiments with formamide concentrations of 10, 20 and 30 % formamide were performed. As a positive control the fluorescence of all organisms was examined by using the domain probe EUB388, which cover the most known bacterial species and all tested organisms, at a formamide concentration of 30 %.

Images, which were used for fluorescence quantification, were taken with Zeiss AxioCam and a constant exposure time of 20000 ms. For Evaluation of images it is important to measure the fluorescence of the single cells and not the fluorescence of larger cell aggregates, as these aggregates show a stronger fluorescence, and hence would falsify results. Fluorescence was measured with DAIME software after fluorescent particles were selected. Fluorescence quantity was measured in Relative Units (RU) For each organism, probe and formamide concentrations at least two separate FISH experiments and a minimum of 200 cells were analyzed.

2.9 Protein determination

Protein was determined using the method of Lowry et al. (1950). Three stock solution were prepared with deionized water and stored at 4°C prior to use: Solution Lowry A contained 2 % (w/v) Na_2CO_3 and a final concentration of 0.1 M NaOH, Lowry B 1 % (w/v) $CuSO_4$ and Lowry C 2 % (w/v) $NaKC_4H_4O_6$* $4H_2O$. Before protein measurement a mixture of the solutions Lowry A, B and C were prepared freshly in a ratio of 99:1:1. For each protein determination a quantum of 0.1 mL sample was pipetted into a 1.5 mL reaction tube, and centrifuged at 15000 rpm for 5 minutes. The resulting pellet was washed in 0.1 mL 1M NaOH. These steps were repeated two times to avoid sulfide residues, that would falsify results of Lowry. The effect of 1 – 5 mM sulfide can be seen in Figure 14. After removal of possible residues 1mL of the mixture was pipetted into the 1.5 mL reaction tube and incubated for 10 min. Then 0.1 mL of Folin Ciolteau Reagent was added and incubated for another 30 minutes. Extinction at 750 nm was determined with a photometer. For calibration dilutions of a bovine serum albumin stock solution (0.5 g per L), which contained 0.0125, 0.025, 0.05, and 0.1 g protein per L) were prepared (Figure 13).

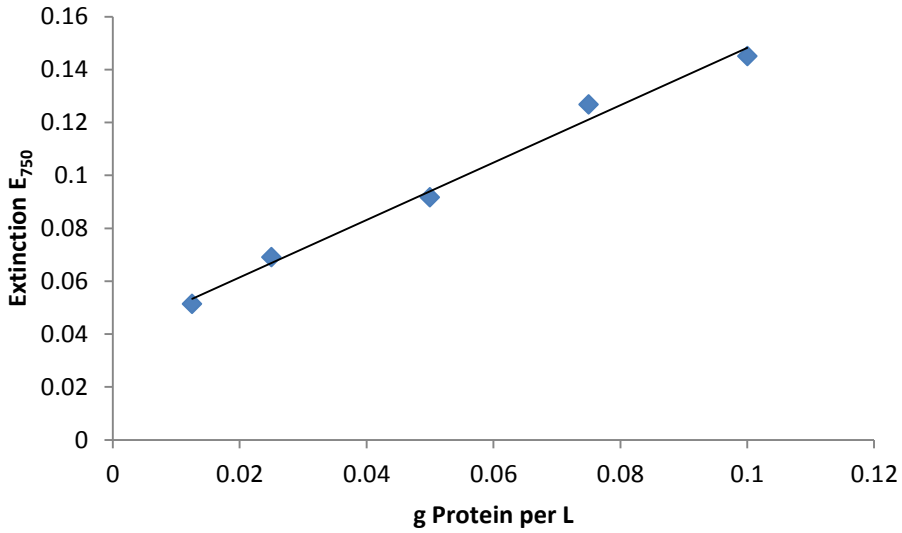

Figure 13: Protein calibration (extinction at 750 nm) curve for different defined standards of bovine serum albumin solutions.

Following equation was determined:

$$Protein\ g\ perL = \frac{Extinction\ (750nm)}{1.0844} - 0.0398;\ R^2 = 0.9909$$

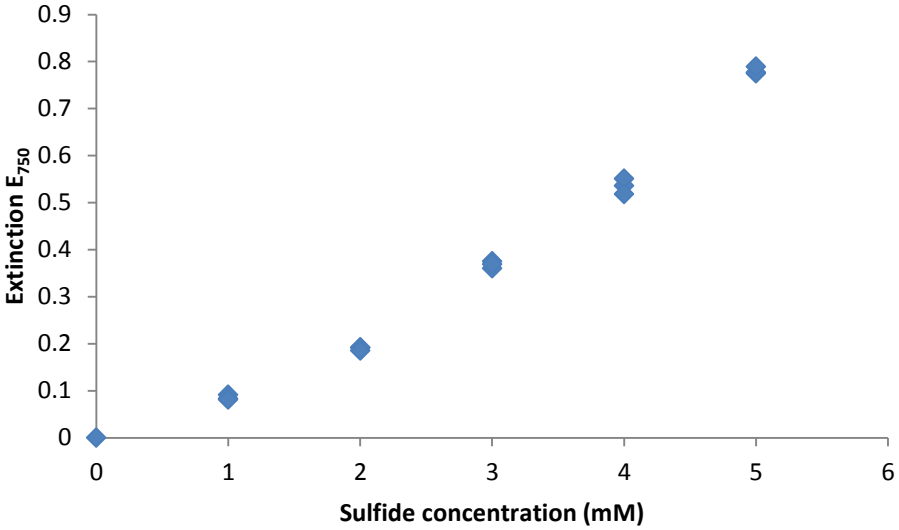

Figure 14: Effect of sulfide on results obtained for protein determination according to Lowry et al. (1950).

3. Results

3.1 Enrichment cultures and co-cultures consisting of two single species

The mesophilic propionate-oxidizing syntrophic enrichment culture from a biowaste reactor was transferred to fresh medium with propionate as sole source of carbon. As the culture was unable to degrade acetate, acetate accumulated 1:1 to the degraded propionate (Figure 15). Additionally the protein content increased also with the production of acetate and the degradation of propionate. Whereas the degradation of the first portion of propionate started after a long lag phase with a slow degradation rate, a rapid degradation of propionate was seen, when propionate was added a second, third or fourth time. In total 29.4 mM propionate was degraded (sum of all propionate additions minus the propionate, which was left at the end of the last addition) and 30.2 mM acetate was produced (Figure 15). The amount of protein increased from 0.016 to 0.173 g per L (Figure 15). Further analysis of data from Figure 15 revealed that after the fourth feeding between day 36 and 43 the culture showed the best degradation abilities, and an average of 0.97 mM propionate per d was degraded. The maximum growth rate was, however, calculated between day 22 and day 28 after the second feeding (0.29 per d) (Figure 15).

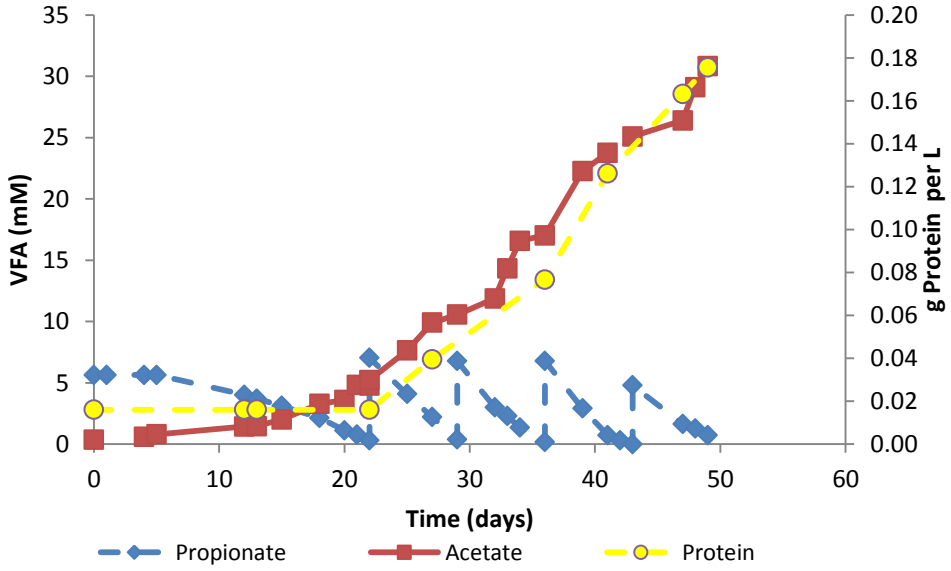

Figure 15: Propionate degradation and production of acetate and protein by a mesophilic propionate-oxidizing syntrophic culture.

A second mesophilic propionate-oxidizing culture contained a vibro-like and a rod-like contaminant. After the second transfer the rod-like contaminant disappeared in all further cultures. Similar to the experiments with mesophilic propionate-oxidizing syntrophic cultures propionate was fed in small portions (Figure 16). In total 25 mM propionate was degraded and 22.8 mM sulfate was reduced during generation of 24.9 mM acetate. Growth in 20 days led to an increase of protein of 0.167 g per L (Figure 16). The highest growth rate was achieved between day 3 and 4 (0.4 per d). Already after the first feeding the degradation of propionate per day was the highest (1.64 mM per d) (Figure 16).

There were also visible differences of the microscopic images between cultures, which degraded smaller amounts of propionate and cultures, which already degraded larger portions. In the second case big aggregates of flocs, containing several hundreds of cells occured (Figure 17).

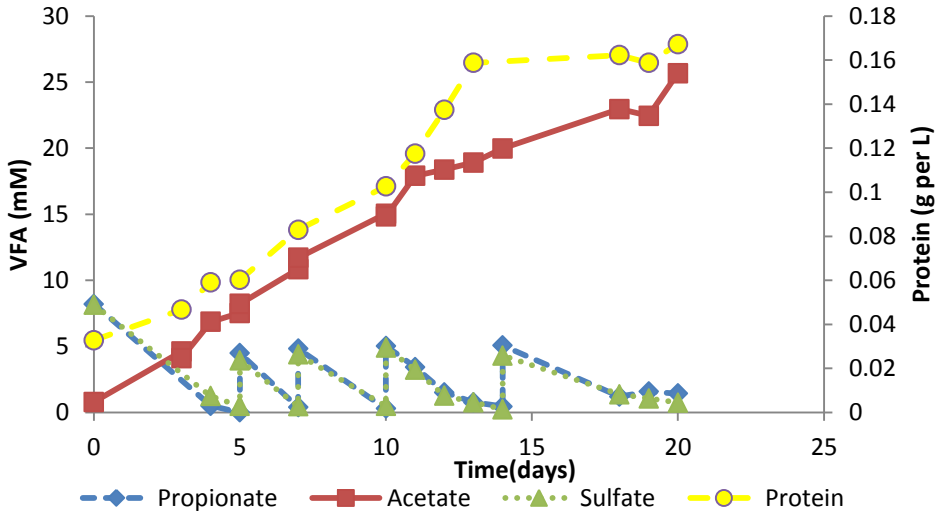

Figure 16: Propionate degradation, sulfate reduction, acetate and protein production of a mesophilic propionate-oxidizing bi-culture.

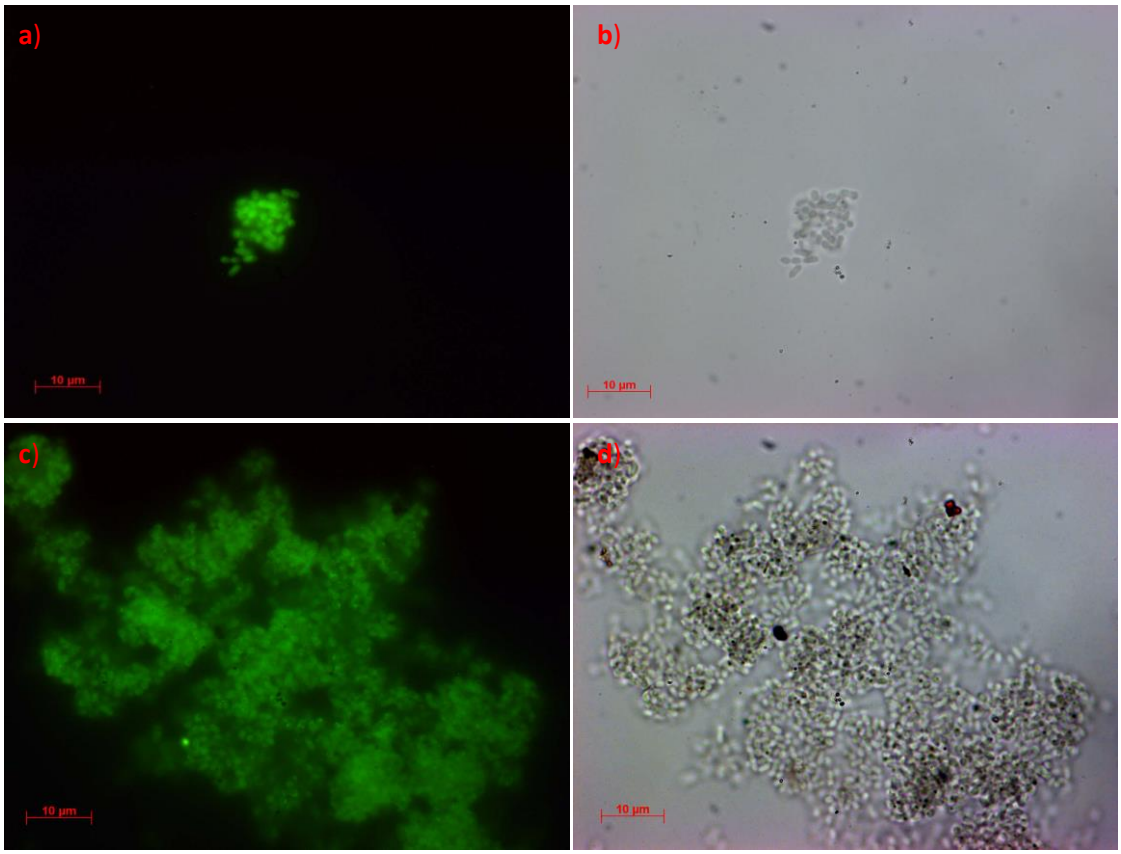

Figure 17: Floc formation of a mesophilic propionate-oxidizing bi-culture. Images of FISH using probe MPOB1 (a, c) and phase contrast microscopy (b,d) after degradation of 0.01 and 2 g propionate.

3.2 Co-digestion of bread with biowaste

Two co-digestion experiments with biowaste as the main substrate were started (Li et al. 2015b). To one reactor in addition to biowaste a white bread suspension was added (Figure 18a), whereas to the other reactor a rye bread suspension was added (Figure 19a). The bread types were selected and separated since rye bread is made with sour dough (containing lactobacilli and thus lactic acid) and white bread is made with yeasts (*Saccharomyces cerevisiae*, performing an ethanol fermentation) (Li et al. 2015b). Both reactors were operated with increasing OLRs by semi continuous feeding of biowaste ± either rye or white bread suspension, and revealed a proportional increasing biogas production at increasing OLR below the maximal possible OLR. Between day 80 and day 105 no VFA were accumulating in reactor 2, when the OLR of the biowaste reactor was increased by addition of increasing amounts of FBS + RBS (Figure 19b), whereas in reactor 1, fed FBS +WBS, an accumulation of propionate and acetate was seen at a slightly decreasing biogas production (Figure 18b). Feeding was stopped in both reactors from day 105 – 115 at overload conditions. After a-starvation period feeding was continued at 22 – 23 kg COD m^{-3} d^{-1}. In both reactors methanogenesis started again within 2 days. In effluent of reactor 1

there was a stable concentration of 1.2 g per L of propionate (Figure 18, 19). A further increase of the OLR by 1 kg COD m^{-3} d^{-1} to 23 – 24 kg COD m^{-3} d^{-1} caused acidification in both reactors. VFA accumulated and the pH dropped below 6, thus feeding was stopped again and the acidic pH was raised to neutral by NaOH addition (Li et al. 2015b). In reactor 1 the recovery of biogas production and of a stable pH above neutral took 7 days longer. The pH remained below 7 for a while and high concentrations VFA including propionate, acetate and n-butyrate were present (Figure 18b). In reactor 2 after the first day of feeding already a pH of 7 could be maintained and only propionate was detected during the first days after re-start of reactor feeding (Figure 19b). Following day 150 at an OLR of 17 kg COD m^{-3} d^{-1} an equal amount of biogas was produced in both reactors with the same gas productivity for this OLR as before the failure, but VFA concentrations in the effluent were still higher and fluctuated heavily up to 3 g per L.

During steady state conditions (days 87 – 101) the numbers of DAPI-stained *Bacteria* and *Archaea* remained stable (Table 11). After the interruption of feeding for digester stabilization a decrease was visible in the number of *Bacteria* at day 115, which continued to fall slowly until day 136 to 0.5×10^9 cells per mL (Figure 20). From day 115 onwards the number of *Archaea* decreased also continuously from 4.0×10^9 cells per mL to 0.3×10^9 cells per mL (Figure 20). In reactor 2 the number of *Bacteria* remained constant for a longer time, as a decrease was only visible in the cell numbers of day 129, when methanogenesis in reactor 2 had failed already. On day 122 with the highest feeding, cell numbers were slightly higher but fell afterwards to 0.5×10^8, when the pH dropped (Figure 20). “After pH correction and resumption of feeding, the population of the *Archaea* recovered” in both reactors (Li et al. 2015b).

Pelotomaculum sp. were the dominant organisms among POB in reactor 1 that was fed biowaste + WBS, contributing up to 85 % to the POB population (Figure 21a, Li et al. 2015b). In the FBS + RBS fed reactor 2 there was a higher diversity of POB, as lower proportions of *Pelotomaculum* sp. (<60 %) versus higher proportions of *Syntrophobacter* (≈20 %) and *Smithella* sp. (≈ 20 %) were detected in the gene probe assays by FISH (Figure 21b, Li et al. 2015). In the recovery phase after overload, when the OLR was raised to 17 kg COD m^{-3} d^{-1} *Syntrophobacter* sp. and *Smithella* sp. disappeared almost completely in both reactors and *Pelotomaculum* sp. represented 100 % of POB (Li et al. 2015b).

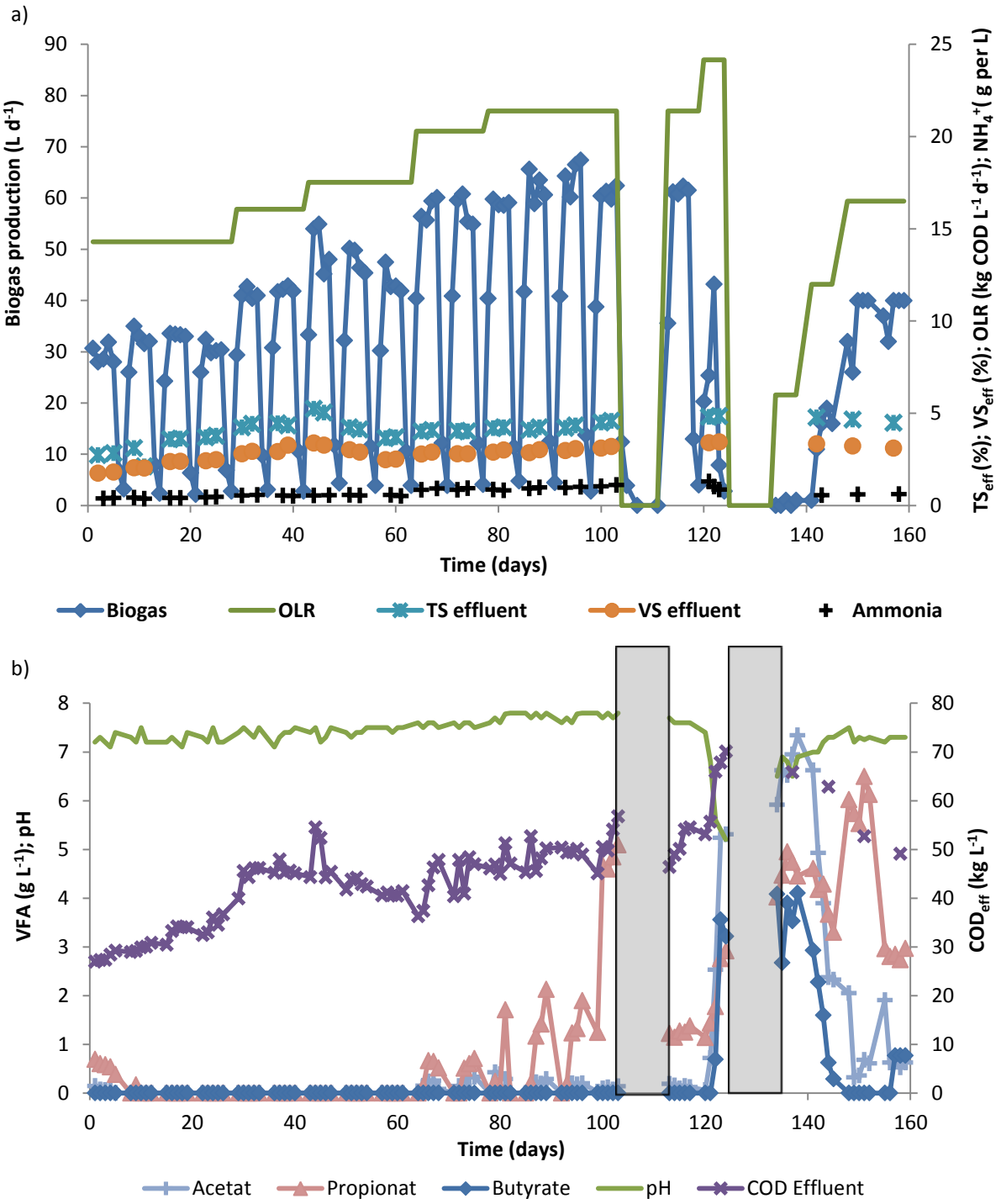

Figure 18: Co-digestion of fresh biowaste suspension (FBS) and wheat bread suspension (WBS). a) Biogas and methane production, TS, OLR and ammonia and b) VFA, pH and COD in the effluent. The grey bars represents phases without feeding. Taken from Li et al. (2015b).

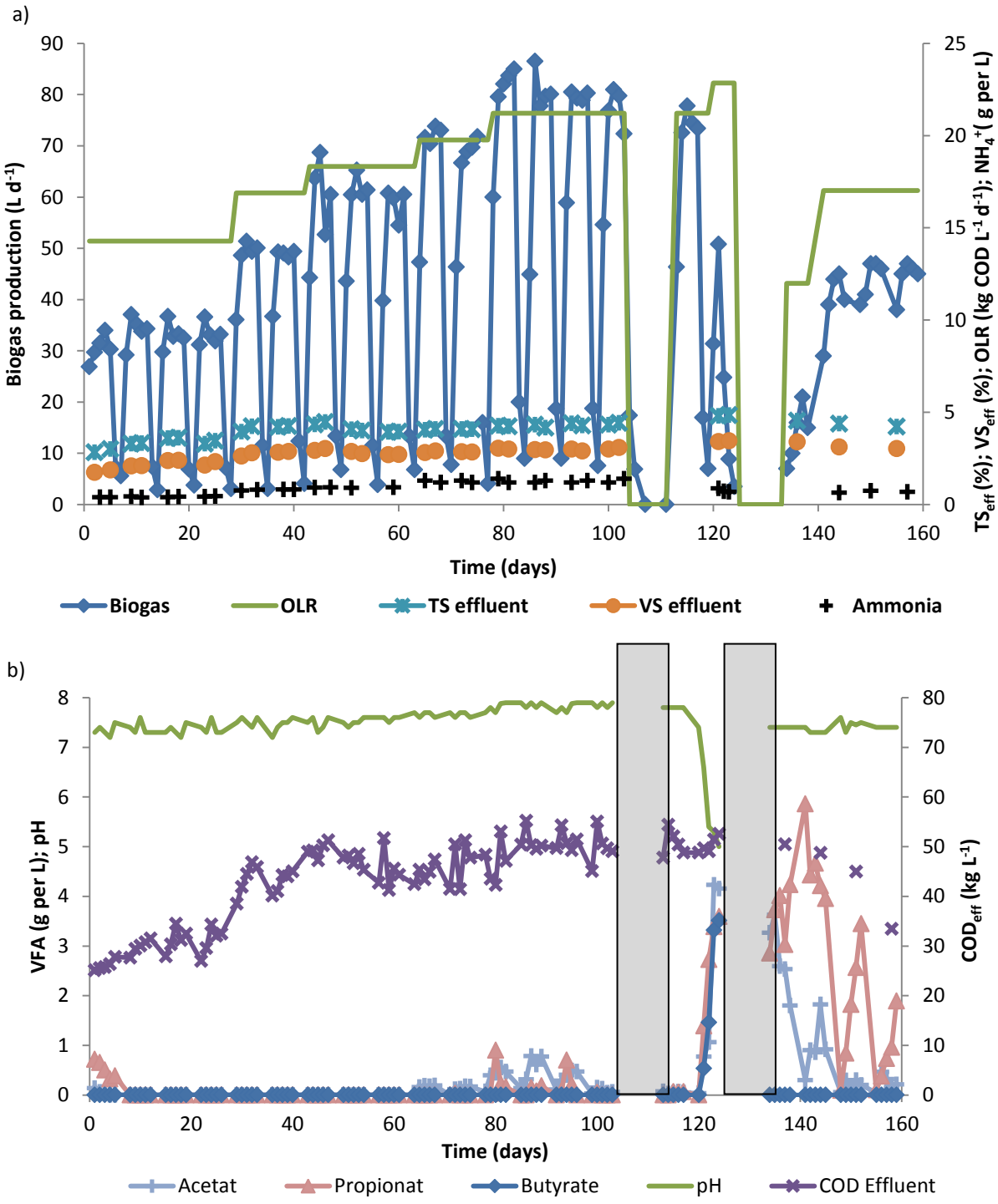

Figure 19: Co-digestion of fresh biowaste suspension (FBS) and rye bread suspension (RBS). a) Biogas and methane production, TS, OLR and ammonia and b) VFA, pH and COD in the effluent. The grey bars represents phases without feeding. Taken from Li et al. (2015b).

Table 11: Counts of DAPI-stained cells, *Bacteria* (Eub388) and *Archaea* (Arc915) during co-digestion of fresh biowaste suspension (FBS) with wheat bread (WBS) or rye bread suspension (RBS). Means and standard deviations (n=10) for mentioned numbers x 10^9 cells.

Time (d)	OLR (biowaste + co-substrate)	Co-substrate	Dapi		*Bacteria* + *Archaea*		*Bacteria*		*Archea*	
			mean	SD		% of DAPI	Mean	SD	mean	SD
87	21.39	wheat (400)	18.4	8,1	11.9	65	10.1	5.5	2.0	1.1
96	21.39	wheat (400)	20.5	1,2	12.9	63	10.5	6.6	2.1	1.6
101	21.39	wheat (400)	19.5	10.7	12.7	65	10.7	5.0,	2.0	1.6
115	21.39	wheat (400)	19.0	6.8	11.8	62	7.8	3,5	4.0	2.2
122	24.16	wheat (500)	14.8	9.3	9.0	61	6.1	4.9	2.9	2.4
129	none	none	11.1	6.0	7.81	70	6.9	4.4	0.9	0.8
136	6,00	none	7.9	7.5	5.53	70	5.2	5.1	0.3	0.3
143	11,99	none	10.3	6.2	7.32	71	6.8	4.5	0.5	0.4
151	17,54	none	19.0	12.8	13.7	72	12.9	9.0	0.8	0.7
158	17,54	none	28.5	4.15	18.8	66	15.1	3.4	2.6	1.6
87	20.34	rye (400)	23.7	10.9	15.2	64	12.8	6.6	2.4	1.5
96	20.34	rye (400)	27.4	12.2	18.2	66	15.4	7.8	2.7	1.9
101	20.34	rye (400)	29.6	18.9	16.0	54	13.1	7.8	2.9	3.2
115	20.34	rye (400)	23.8	13.6	14.9	63	11.2	7.2	3.7	2.7
122	22.85	rye (500)	22.6	8.7	15.4	68	14.1	7.9	1.3	0.8
129	none	none	12.2	7.8	9.7	79	8.0	5.8	1,7	1,4
136	11.99	none	7.0	6.4	5.1	73	4.7	3.9	0.5	0.5
143	17.02	none	12.4	9.5	9.4	75	8.5	6.3	1.8	2.3
151	17.02	none	16.9	9.4	10.8	64	6.2	4.3	3.5	2.3
158	17.02	none	21.8	6.3	14.7	67	10.0	2.8	4.4	1.3

The vast majority of methanogens in the co-digesters of biowaste (FBW + RBS or FBW + WBS) were members of the order *Methanomicrobiales.* In FBW + WBS fed reactor 1 numbers of methanogens were between 1.4 x 10^9 and 1.9 x 10^9 cells per ml at steady state and in FBS + RBS fed reactor 2 even higher, between 2.1 x 10^9 and 2.5 x 10^9 cells per ml (Figure 22). When the reactors finally failed at high OLR, numbers of methanogens were reduced like for all members of the methanogenic mixed population at this time. The population of *Methanomicrobiales* recovered faster in reactor 2 (Figure 22). Members of *Methanosarcinales* contributed only a small minority to the methanogens in both reactors (Figure 22). Between day 80 – 120 there was an increase of ammonia in reactor 1 fed FBS + WBS . Ammonia levels increased from below 1 to 1.3 g per L, causing a slight decrease of the population density of *Methanosaeta* sp. (Figure 23). In digester 2, that was fed FBS + RBS the ammonia concentration was between 1.2 and 1.4 g per L, hence small numbers of

Methanosaeta sp., around 3 x 10^6 cells per ml, were present. At day 121, when the ammonia concentration fell below 1 g per L the population density of *Methanosaeta* increased to numbers of 1.2 x 10^8 cell per mL (Figure 23).

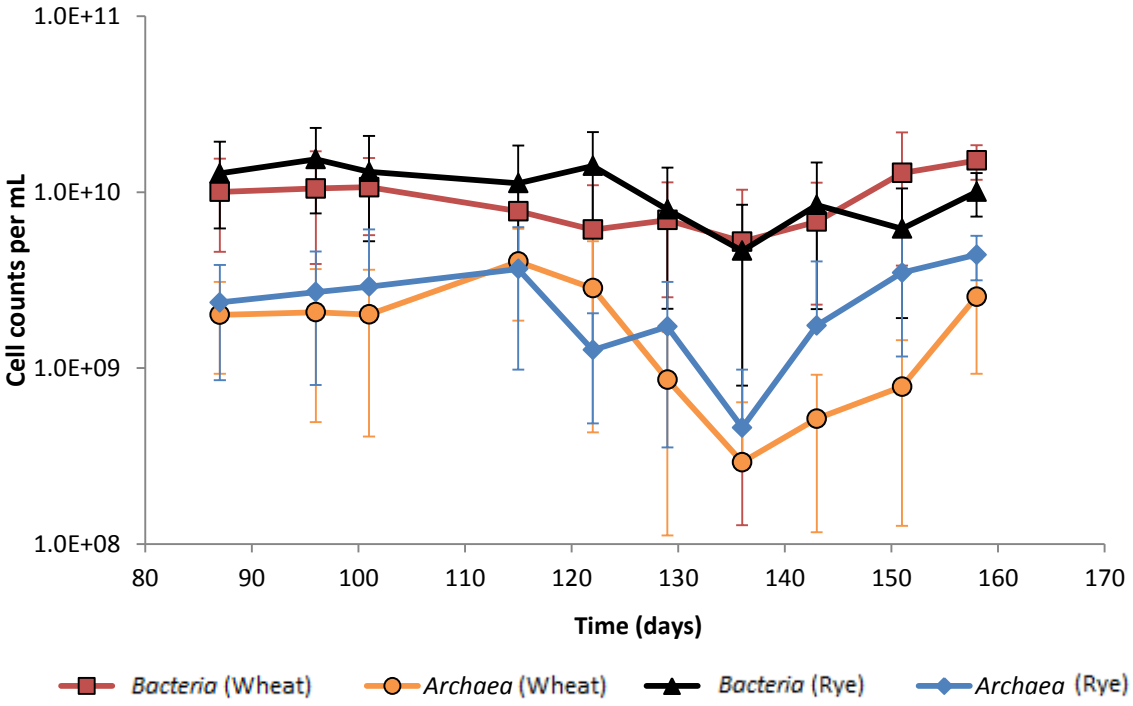

Figure 20: Population density changes of *Bacteria* and *Archaea* during co-fermentation of wheat and rye bread suspensions in a biowaste digester. Taken from Li et al. (2015b).

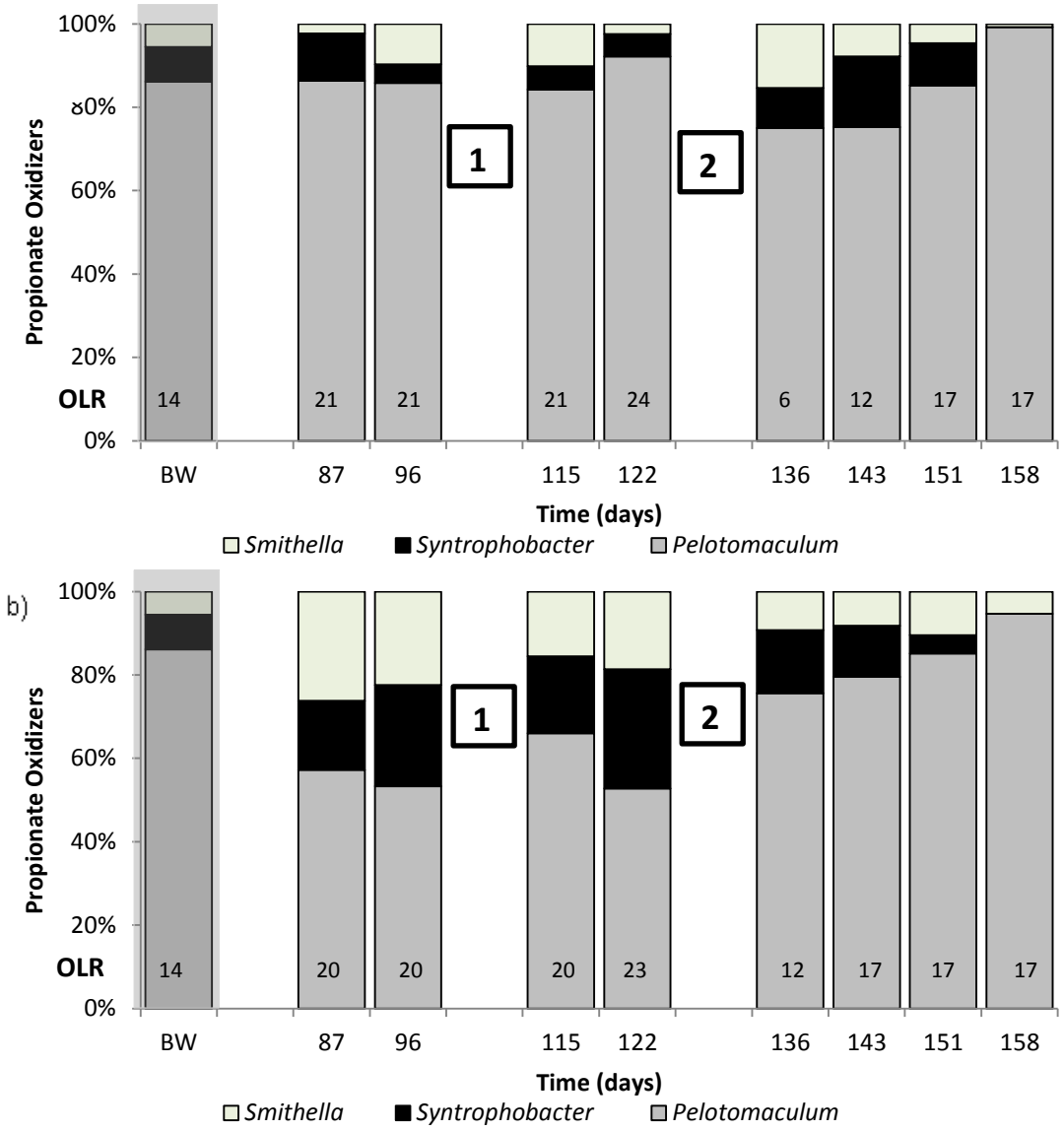

Figure 21: Proportions of POB in the reactors with wheat bread (a) and rye bread (b). 1: No feed for 1 week, fine sand removal after clogging. 2: No feed for 1 week due to overload. Numbers in column represent OLR at sampling. BW = biowaste. Taken from Li et al. (2015b).

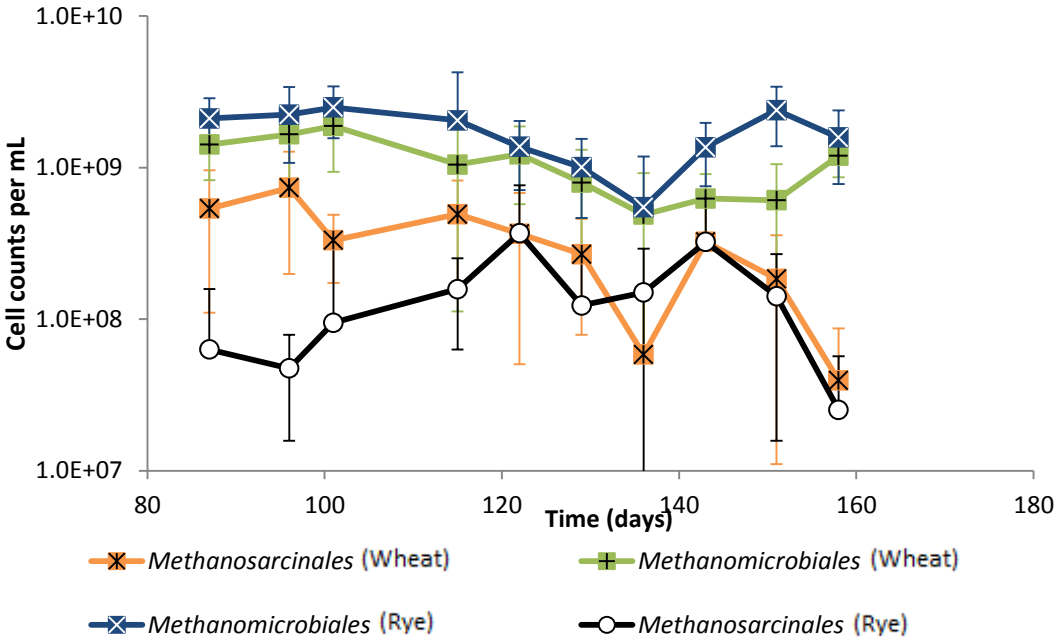

Figure 22: Number of *Methanosarcinales* and *Methanomicrobiales* in reactor 1 FBS+WBS and reactor 2 FBS+RBS. Means and standard deviations (n=10). Adapted from Li et al. (2015b).

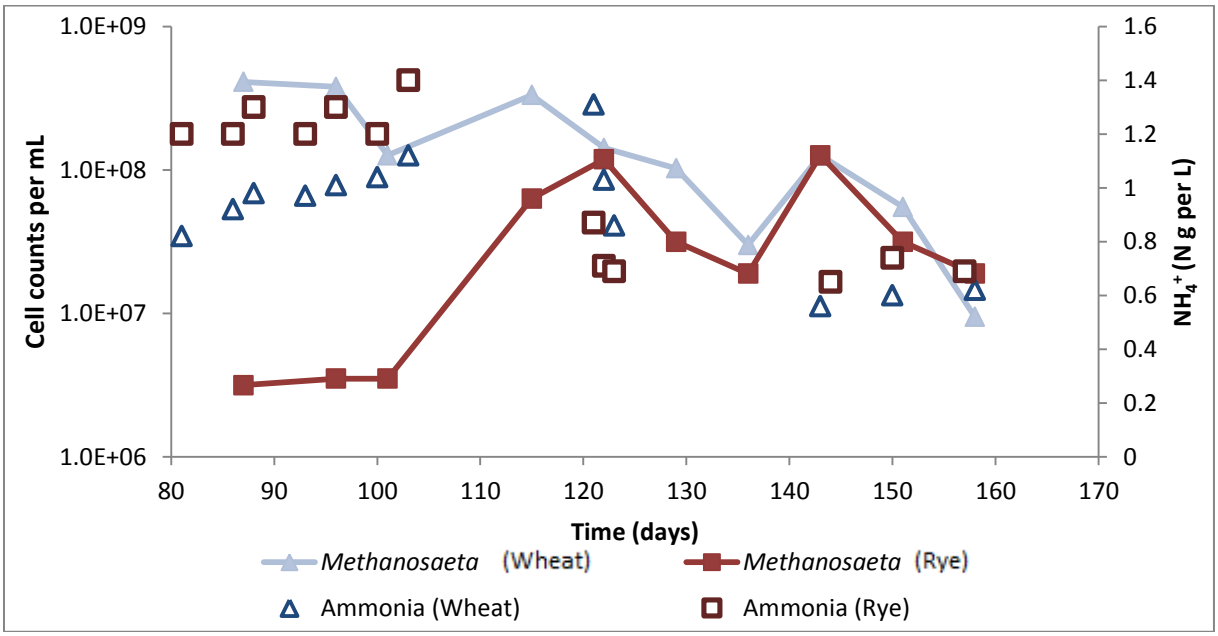

Figure 23: Development of *Methanosaeta* sp. during wheat bread and rye bread co-digestion with biowaste suspension. Taken from Li et al. (2015b).

3.3 Full scale re-start after revision

Concerning VFA concentrations in the suspension of the full scale biowaste digestion plant Karlsruhe-Durlach two phases were distinguished: A phase of acidification, where acetate accumulated during 6 days after re-start-up to 1.3 g per L and propionate during the first 8 days to 4.6 g per L (Figure 24). Then, in a second phase within the following 5 days a complete degradation of propionate and acetate took place, and no acetate or propionate was detected further on. Concentrations of n-Butyrate remained below the detection limit of the used GC

Figure 24, Moertelmaier et al. 2014).

In biowaste samples, that were collected from the digester of the full scale biowaste treatment plant in Karlsruhe Durlach during start-up after a major revision, where the digester was completely emptied, an increase of DAPI-stainable cell counts representing *Bacteria* and *Archaea*, could be seen. The number of DAPI-stainable cell counts increased continuously from 0.5×10^{10} to 1.5×10^{10} cells per mL within the first 13 days after re-start. Then cell numbers stabilized between 1.0 and 1.3×10^{10} cells per mL from day 14 till 19 (Table 12). In the long term perspective (81, 111, and 181 days after re-start) DAPI-stainable cell numbers were between 0.7×10^{10} and 1.1×10^{10} cells per mL (Table 12). The number of DAPI-stainable cells in inocula was equal to the number of cells found at day 0 after re-start (Table 12). There is a gap between the sum of *Bacteria* and *Archaea* numbers using specific gene probes and DAPI-stainable cell numbers. The sum of *Bacteria* and *Archaea* added up to maximally two third of counted DAPI cells. For changing digester conditions, in general the numbers of *Bacteria* + *Archaea* together show a similar development towards lower or higher values as DAPI numbers. There were, however, slight differences visible when *Bacteria* and *Archaea* were compared separately (Table 12). Cell numbers of *Bacteria* increased earlier than the numbers of *Archaea*. *Bacteria* approximately tripled from $(1.6 \pm 1) \times 10^9$ to $(4.4 \pm 2.4) \times 10^9$ cells per mL, with a variation between $(3.7 \pm 1.5) \times 10^9$ and $(5.4 \pm 2.5) \times 10^9$ cells per mL (Figure 25, Moertelmaier et al. 2014). At day 10 the population of *Archaea* was still at $(0.8 \pm 0.3) \times 10^9$ cells per mL, but then *Archaea* began to grow within 4 days to $(3.0 \pm 0.6) \times 10^9$ cells per mL, which means an increase by a factor of 2.75 within 4 days. In the last days of the start-up phase archaeal cell numbers were around 2.0×10^9 cells per mL and later on during steady state cell numbers fluctuated between $(1.2 – 1.9) \times 10^9$ cells per mL. At the first day after re-start the number of *Bacteria* was 1.6×10^9, about two times higher than the numbers of *Archaea* with 0.8×10^9 cells per mL. In the time of maximal growth of *Bacteria* and fatty acid accumulation the proportion of *Archaea* fell to 16 % at day 7, whereas afterwards in the time of the maximal archaeal growth and fatty acid degradation the proportion of *Archaea* reached 38 %, e.g. at day 13 (Figure 26, Moertelmaier et al. 2014).

On the first day of re-start of the biowaste reactor the number of all POB was 2.2×10^8 cells per mL, which means a contribution of POB to total *Bacteria* of 7.5 % (Figure 27). *Pelotomaculum* sp. dominated the POB and contributed between 74 % and 95 % of all POB. The rest were *Syntrophobacter* sp. and *Smithella* sp., with changing proportions (Figure 28).

Within the first 3 days of re-start the numbers of *Pelotomaculum* sp. and *Smithella* sp. in the biowaste reactor remained stable, but at day 5, when acetate and propionate concentrations began to increase, numbers of *Pelotomaculum* sp. decreased by 72 % from $(1.8 \pm 1.1) \times 10^8$ to $(5 \pm 7) \times 10^7$ cells per mL and numbers of *Smithella* sp. by 97 % from 5×10^7 to 1.6×10^6 (Figure 27, Moertelmaier et al. 2014). Both *Smithella* and *Pelotomaculum* sp. recovered in the next days, *Pelotomaculum* sp. to 3.8×10^8 cells per mL at day 13. At steady state conditions including the long term measurements after 81, 111, and 181 days numbers of *Pelotomaculum* sp. remained between $(1.2 \pm 0.8) \times 10^8$ and $(2.8 \pm 2) \times 10^8$ cells per mL. *Smithella* sp. reached numbers of 1×10^7 cells per mL when acetate was already decreasing, but propionate still increased. At steady state conditions numbers were lower again but clearly above the detection limit (Moertelmaier et al. 2014). The numbers of the *Syntrophobacter* population showed a different development. *Syntrophobacter* sp. increased from $(0.5 \pm 0.8) \times 10^7$ to $(2 \pm 2.6) \times 10^7$ cells per mL during propionate accumulation and its subsequent degradation (Moertelmaier et al. 2014). During steady state *Syntrophobacter* sp. decreased to its initial density in the inoculum (Figure 27). Long after re-start of the reactor (days 81, 111, 181) numbers of *Syntrophobacter* sp. were quite stable between $(2.2 \pm 2.1) \times 10^7$ and $(2.5 \pm 2.5) \times 10^7$ cells per mL (Moertelmaier et al. 2014).

The methanogenic community consisted of members of the order *Methanosarcinales* including high numbers of *Methanosaeta* sp. and *Methanomicrobiales*. Their proportion, however, changed by time. Except for the 2 taxa, which were the main methanogenic bacteria in the biowaste digester, a few *Methanobacteriales* were also found, but they may not have had a significant effect on biogas production. Fluctuating numbers of between $(0.3 \pm 0.6) \times 10^7$ and $(2.7 \pm 0.4) \times 10^7$ cells per mL were *Methanobacteriales*. At some day their number was even below the detection limit of 1.6×10^6 cells per mL.

As *Methanosaeta* sp. is a major acetate-converting organism within the order of *Methanosarcinales*, it is not surprising that the number of cell counts was related to acetate levels (Moertelmaier et al. 2014). *Methanosarcinales* and *Methanosaeta* sp. stopped to grow at day 5 and cell numbers decreased, when, presumably due to high load, propionate and acetate production rates increased over their degradation rates. Both groups recovered and degraded acetate, accompanied by an increasing population of $(0.7 – 1) \times 10^9$ cells per mL (Figure 29). The hydrogenotrophic *Methanomicrobiales* seem to have an important role during start-up, as they showed a more than 5-fold increase of cell numbers from $(1.6 \pm 1.2) \times 10^8$ to finally $(8 \pm 4.5) \times 10^8$ cells per mL. The most rapid growth occurred in the phase, when propionate and acetate were degraded (Figure 29, Moertelmaier et al. 2014). The highest proportion of Methanomicrobiales could be found in the undigested substrate, whereas the proportion of Methanosaeta sp. is very high within the first days after re-start (Figure 30). Till day the proportion of Methanomicrobiales recovered to > 40 %. In the long term observation (days 81, 101, 181) proportion was lower again, however(Figure 30).

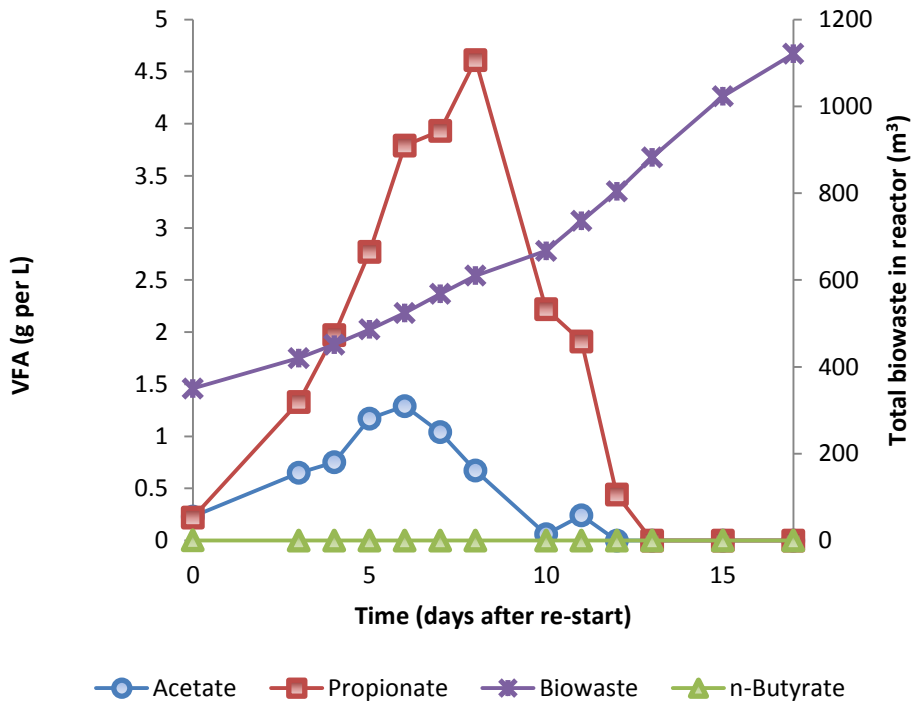

Figure 24: Volume of biowaste and VFA concentrations in the full scale biogas reactor Karlsruhe Durlach during start-up. Taken from Moertelmaier et al. (2014).

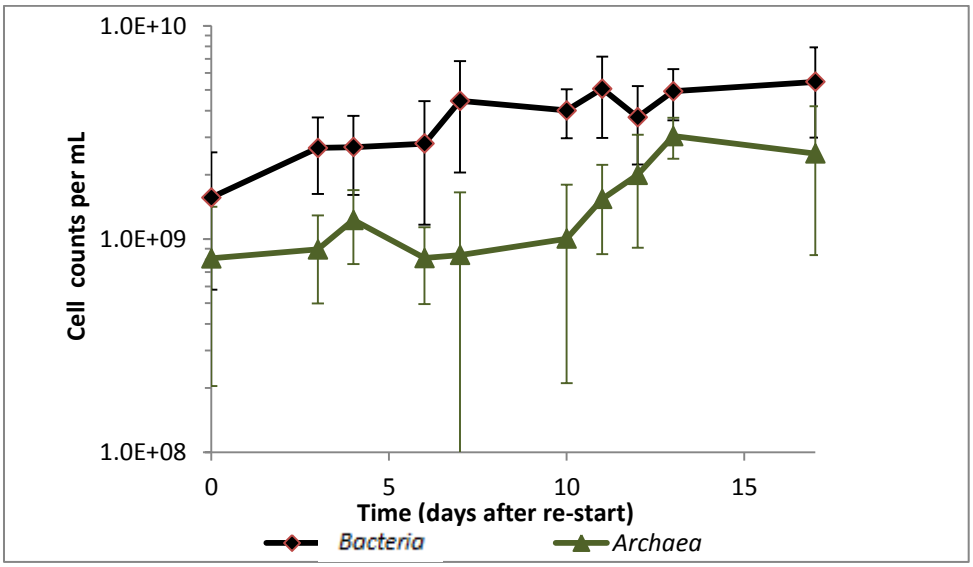

Figure 25: Number of *Bacteria* and of *Archaea* during start-up of the full-scale reactor after revision (n=10). Taken from Moertelmaier et al. (2014).

Table 12: Counts of DAPI *Bacteria* (Eub388) and *Archaea* (Arc915) during start-up of the full-scale reactor after revision. Means and standard deviations (n=10) for mentioned numbers x 10^8 cells.

	DAPI		*Bacteria+Archaea*		*Bacteria*		*Archaea*	
	Mean	SD		% of DAPI	mean	SD	mean	SD
Substrate	36.2	27.3	25.6	71	19.1	17.4	6.5	9.9
Inoculum	54.1	26.8	39.1	72	29.8	18.4	9.3	4.9
Days after re-start								
0	52.6	18.4	23.8	45	15.6	9.9	8.1	6.1
3	61.0	18.9	35.7	58	26.7	10.5	8.9	4.0
4	72.6	16.6	39.2	54	27.0	10.8	12.3	4.7
6	58.2	21.1	36.2	62	28.0	16.4	8.1	3.2
7	85.2	51.1	52.8	62	44.4	23.9	8.4	8.1
10	90.0	24.6	50.1	56	40.0	10.4	10.0	7.9
11	106.2	38.8	66.1	62	50.7	20.9	15.4	6.9
12	110.0	43.0	57.2	52	37.2	14.9	20.0	10.9
13	147.6	33.2	79.7	54	49.3	13.3	30.4	6.6
14	122.7	46.1	65.1	53	46.7	25.2	18.3	5.2
17	132.5	56.7	79.8	60	54.6	24.7	25.2	16.8
18	121.0	44.8	65.6	54	45.0	21.2	20.6	10.0
19	114.0	35.5	64.4	57	44.5	18.1	19.9	10.7
81	81.8	26.4	51.0	62	32.4	12.5	18.6	5.1
111	76.5	16.0	39.2	51	27.7	15.0	11.5	4.0
181	106.2	21.6	57.2	54	44.0	14.5	13.3	6.3

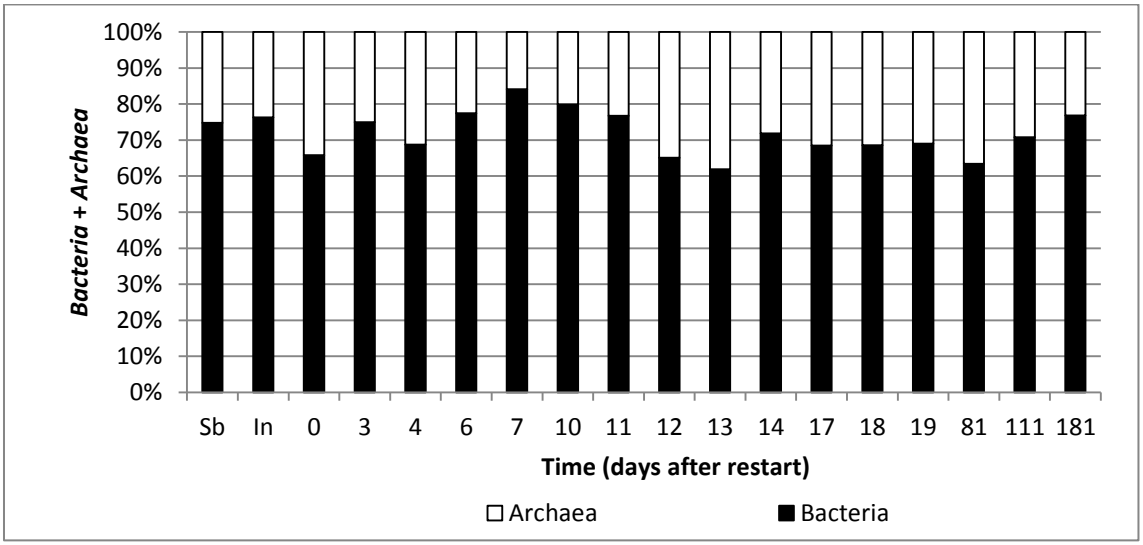

Figure 26: Proportions of *Bacteria* and *Archaea* of the full-scale reactor after revision. Adapted from Moertelmaier et al. (2014).

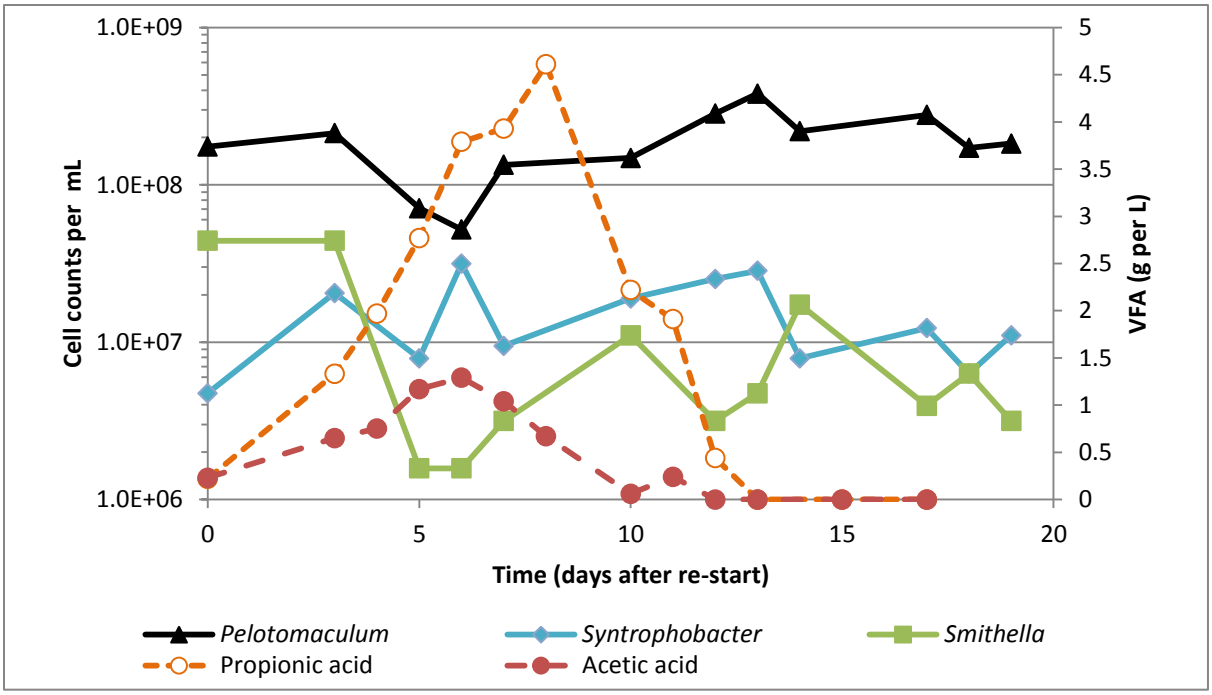

Figure 27: Numbers of POB during start-up and concentration of propionic acid and acetic acid. Taken from Moertelmaier et al.(2014)

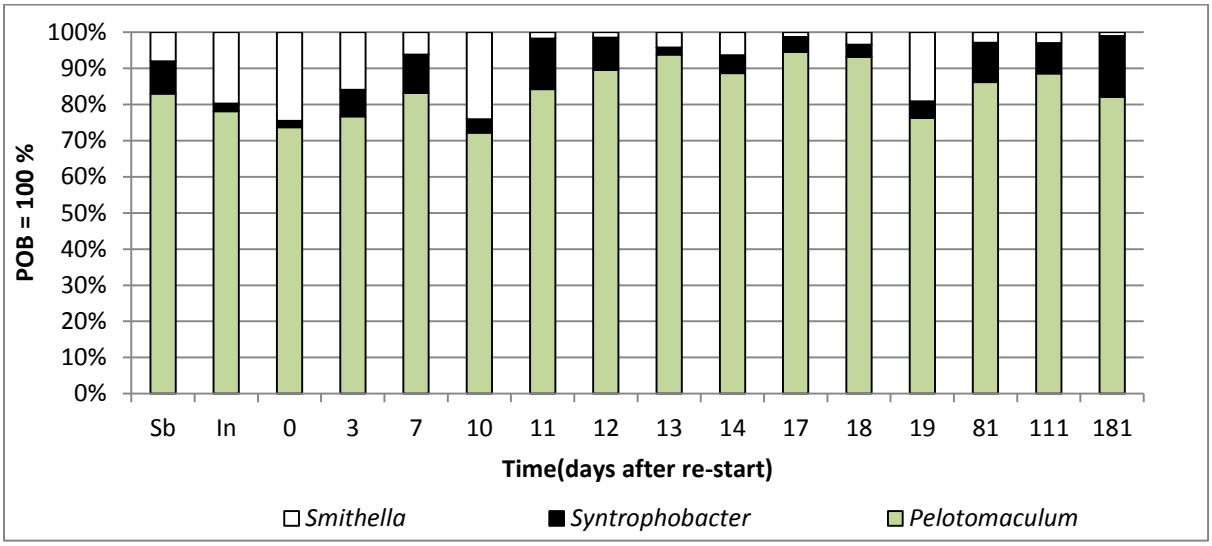

Figure 28: Proportions of POB during start-up phase (0 – 19 d) and in the long term observation 81 – 181 d. Taken from Moertelmaier et al. (2014).

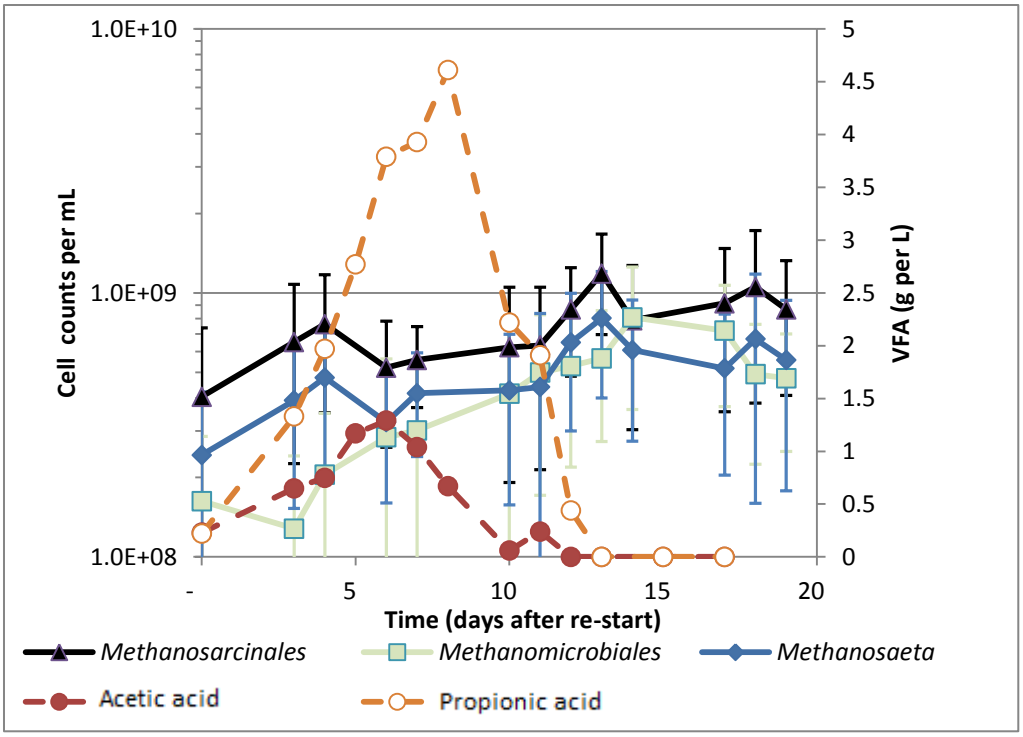

Figure 29: Numbers of relevant methanogenic orders and concentrations of acetate and propionate during start-up. Taken from Mörtelmaier et al. (2014).

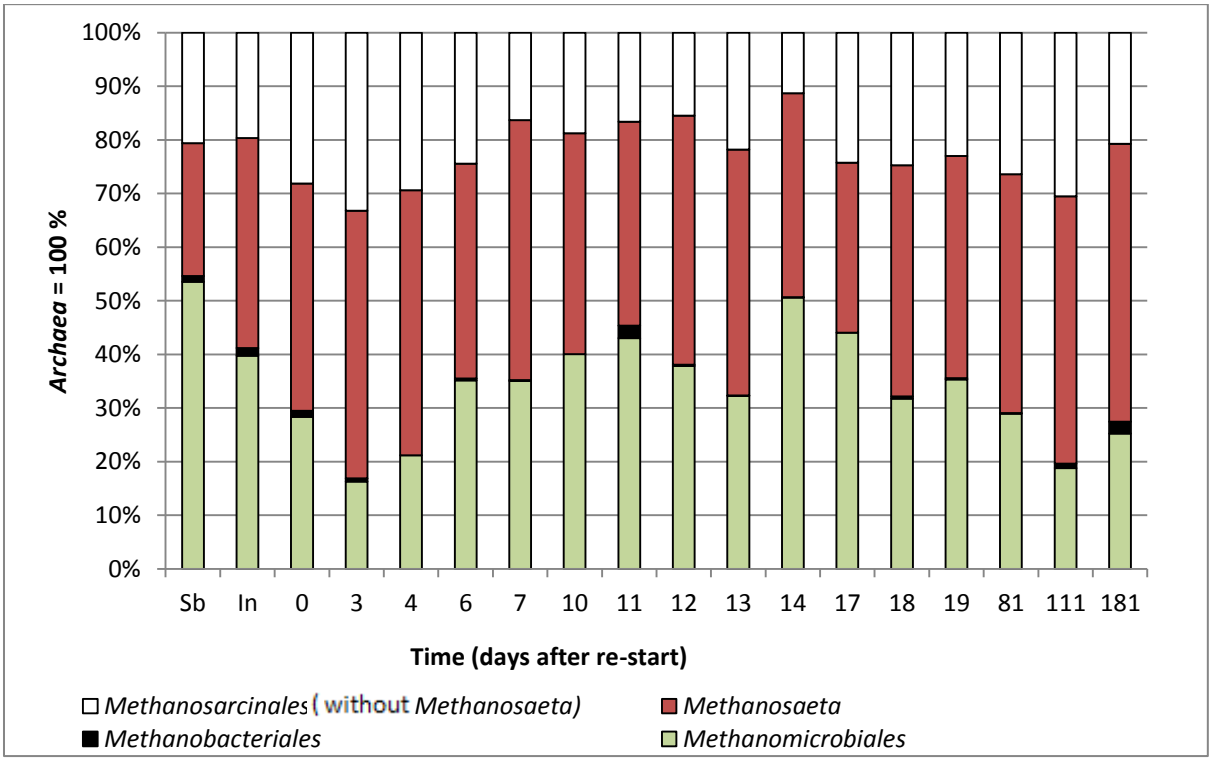

Figure 30: Proportions of methanogenic taxa during start-up phase (0 – 19 d) and in the long term observation from day 81 to day 181. Taken from Mörtelmaier et al. (2014).

3.4 Propionic acid co-digestion with biowaste

Propionic acid co-digestion together with a biowaste suspension was performed in three reactors: Reactor 1 (run 1) was a simulation of the full scale digestor plant of Karlsruhe Durlach. Hence the reactor was fed with biowaste suspension alone, without any further addition of propionate. Feeding was done Monday till Friday, which leads to an OLR of 12 kg COD m^{-3} d^{-1} (Li et al. 2015a). Within the first three weeks propionate accumulated between Monday and Friday. During weekends, when no biowaste was added, all accumulated propionate was degraded (Figure 31). Steady state conditions were reached 25 – 27 days after start of the reactor, when methanogenic activity reached its maximum and no VFA accumulation could be detected (Figure 31). The stop of feeding during weekends led to a decrease of biogas production from average 34 L d^{-1} from Tuesday to Friday to less than 5 L d^{-1} until Monday morning. "After resuming biowaste feeding, on Mondays biogas generation (Figure 31, days 8, 15, 22, 29, 36, 43) was much lower than on Tuesday till Friday, indicating metabolic limitations. Only about 35 days after start was the metabolic activity of the community stable and high enough so that after starvation and resuming feeding on Monday only slightly less biogas (28 – 30 L d^{-1}) was produced than from Tuesday to Friday (31 – 35 L d^{-1})" (Li et al. 2015a).

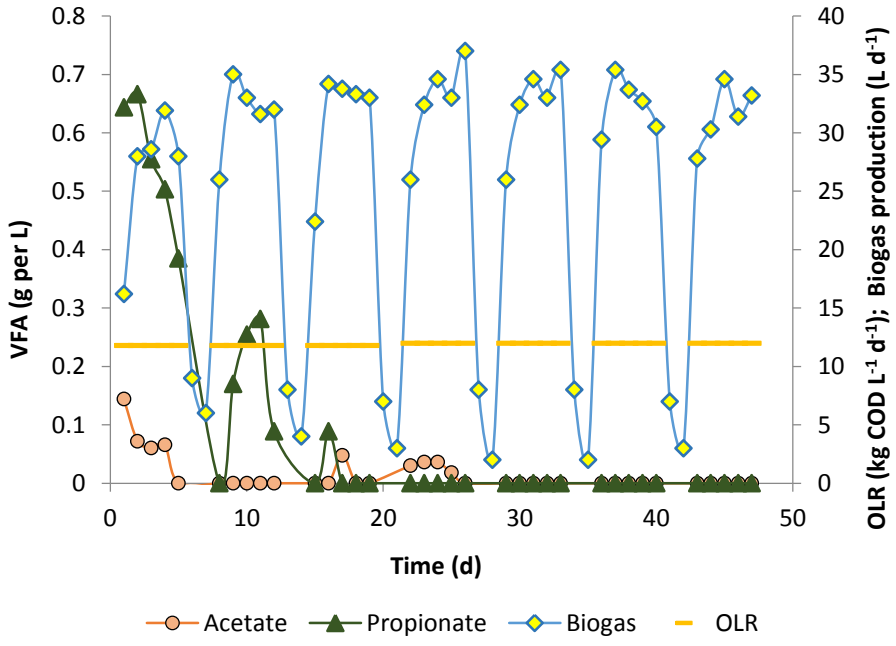

Figure 31: Biogas production and fatty acid levels in an 10 L biowaste digester (run 1) after start at an OLR of 12 kg $COD_{biowaste}$ m^{-3} d^{-1}. Taken from Li et al. (2015a).

Reactor 2 (run 2) was operated with a basic OLR of 12 (day 1 – 55) or 14 kg $COD_{biowaste}$ m^{-3} d^{-1} (day 55 onwards) and increasing amounts of propionate (Li et al. 2015a). As reactor 1 reactor 2 was fed from Monday till Friday, so Saturday and Sunday were again phases of starvation. The OLR was increased stepwise to 18 kg COD m^{-3} d^{-1} by co-feeding propionate (Figure 32). Propionate accumulation was seen after every increase of the OLR during the week. The propionate concentration was even higher than the added propionate. During

the starvation periods on weekends accumulated propionate was degraded. In the second week after an increase of the OLR by propionate, propionate concentrations were already lower than in the first week, indicating some kind of adaptation. Propionate concentrations decreased even further in the third week after an OLR increase (Li et al. 2015a).

With increasing OLR, gas production increased with lower values on Monday than from Tuesday to Friday (Figure 32, days 0 – 75), indicating some activity losses during starvation on weekends without feeding. During 10 days interruption of the feeding for glass repairs (Figure 32, day 75 – 85), POB lost much of their metabolic activity (Li et al. 2015a). After resuming biowaste + propionate feeding at almost the same OLR as before maintenance, when no propionate was detected in the digester effluent, the highest propionate peak at all was measured (Figure 32, day 89). Complete regeneration of the propionate degradation activity by POB took more than 40 days. Finally, at the very high OLR of 18 kg COD m^{-3} d^{-1}, maintained with biowaste + propionate feeding for 5 days per week with no feeding on Saturday and Sunday, steady state conditions without residual fatty acids in the effluent were obtained (Figure 32, days 130 – 140 and further), (Li et al. 2015a).

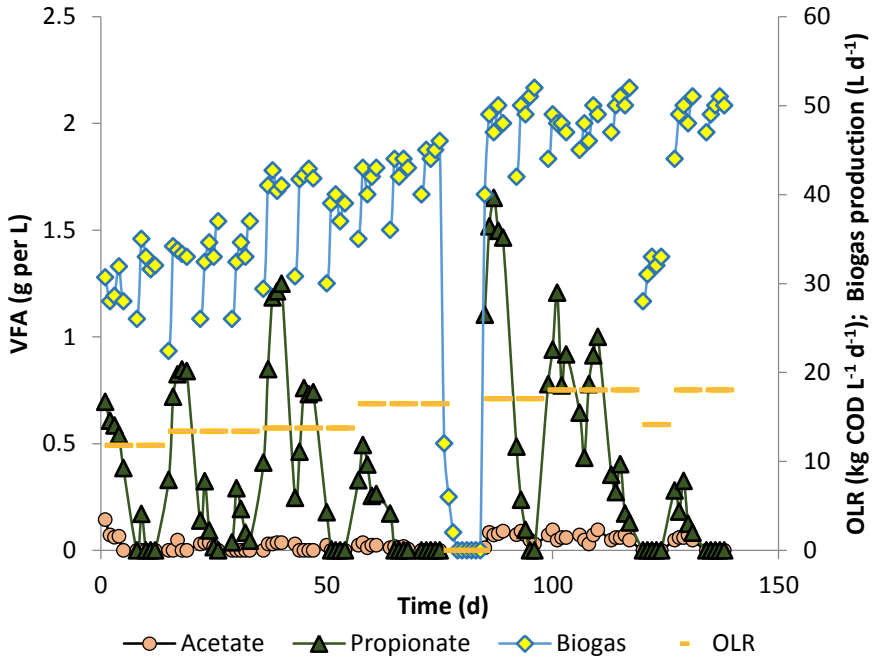

Figure 32: Biogas production and fatty acid levels in an 10 L biowaste digester (reactor 2) for increasing organic loading rates up to 18 kg COD m^{-3} d^{-1}, maintained by 12 kg (day 1 – 55) or 14 kg (new batch biowaste from day 55 onwards) $COD_{biowaste}$ m^{-3} d^{-1} plus respective amounts of propionate. No feeding between days 75 – 85 due to maintenance works. Taken from Li et al. (2015a).

After re-starting methanogenesis by the feed of biowaste alone for 4 days, reactor 3 (run 3) was operated with a continuous inflow of propionate (3 kg $COD_{propionate}$ m^{-3} d^{-1}) (Li et al. 2015a). From Monday to Friday biowaste suspension was additionally added, which led to an increase of the OLR from 3 to 14 kg COD m^{-3} d^{-1} (Figure 33). Biogas production from propionate alone during weekends was relatively low (16 – 18 L d^{-1}), compared to the

amount of biogas (40 L d^{-1}), that was produced when fresh biowaste was fed (Figure 33). Every time when fresh biowaste was added on Mondays propionate levels increased. After the third weekend of propionate addition no propionate could be detected in the effluent. A stable level of acetate was found in the effluent, which disappeared completely after day 22 (Li et al. 2015a), (Figure 33).

Feeding was stopped between days 25 and 28 and a new feeding regime was introduced from day 29 till 43 (Li et al. 2015a). The reactor was run with biowaste feeding at an OLR of 11 kg COD m^{-3} d^{-1} from Monday until Friday. During weekends (from Friday night to Monday morning) propionate was added at an OLR of 5 kg COD m^{-3} d^{-1} (Figure 33). The continued feeding of propionate instead of biowaste suspension from Friday to Monday led to a very stable biogas production, varying between 25 – 28 L d^{-1}. No accumulation of VFA could be detected in the digester effluent at any time. "Thus the fermentation was stable, representing steady state conditions" (Li et al. 2015a).

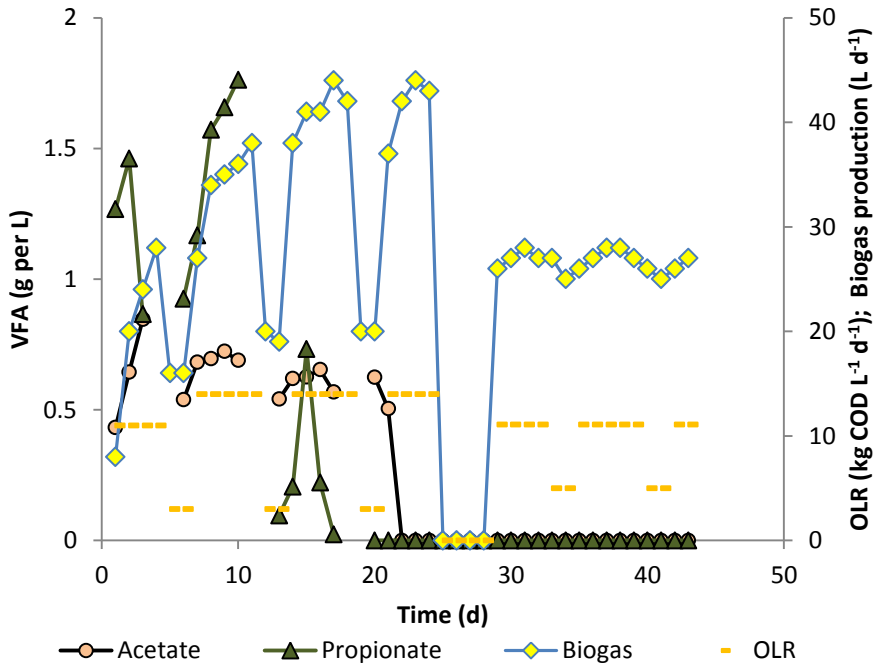

Figure 33: Biogas production and fatty acid levels in an 10 L biowaste digester (reactor 3) for 2 different feeding modes after reactor re-start with biowaste alone (11 kg COD m^{-3} d^{-1}) within the first 4 days. In the first phase 3 kg $COD_{propionate}$ m^{-3} d^{-1} was fed continuously and additionally 11 kg $COD_{biowaste}$ m^{-3} d^{-1} was fed from Monday till Friday (in total 14 kg $COD_{biowaste}$ m^{-3} d^{-1}). In the second phase biowaste was fed from Monday till Friday and propionate during weekends. No feeding between days 25 – 28 due to maintenance works. Adapted from Li et al. (2015a).

Numbers of *Bacteria* increased from (2.7 ± 1.6) × 10^9 to (5.2 ± 3.5) × 10^9 per mL within the first 24 days. Later on numbers varied between(3.4 ± 0.8) × 10^9 and (4.9 ± 1.3) × 10^9 per mL. The number of *Archaea* remained below 1 × 10^9 per mL at first, but exceeded 1 × 10^9 per mL in the last 14 days of operation (day 38 and 45; Figure 34a). Numbers of POB increased from 0.7 to 2.9 × 10^8 per mL between day 16 and day 24. Afterwards numbers decreased, but

remained above 1.0×10^8 per mL (Figure 34b). Most POB were *Pelotomaculum* sp. After day 16 *Pelotomaculum* sp. numbers represented the number of total POB and were always above 1.0×10^8 per mL. *Syntrophobacter* sp. could be detected at all sampling times and cell numbers were around 1.0×10^7 per mL. Thus 10 % of POB were *Syntrophobacter* sp. Hardly any cells of *Smithella propionica* could be found, hence this organism did not seem to play any role in our reactors (Figure 34c). *Methanomicrobiales* had the highest numbers of cells among all tested subgroups of methanogens at every sample time. At day 16 and 19 numbers were low (5.2×10^8 per mL), later on numbers increased and varied between $(6.4 \pm 5.1) \times 10^8$ per mL and $(9.1 \pm 6.5) \times 10^8$ per mL (Figure 34d). Less numerous but still relevant numbers of *Methanosarcinales,* including *Methanosaeta* sp.*,* were also present. After 31 days of operation numbers of *Methanosarcinales* were between 3.7×10^8 per mL and 5.7×10^8 per mL and numbers of *Methanosaeta* between 2.8 and 3.8×10^8 per mL, respectively (Figure 34d). *Methanobacteriales* could hardly be found.

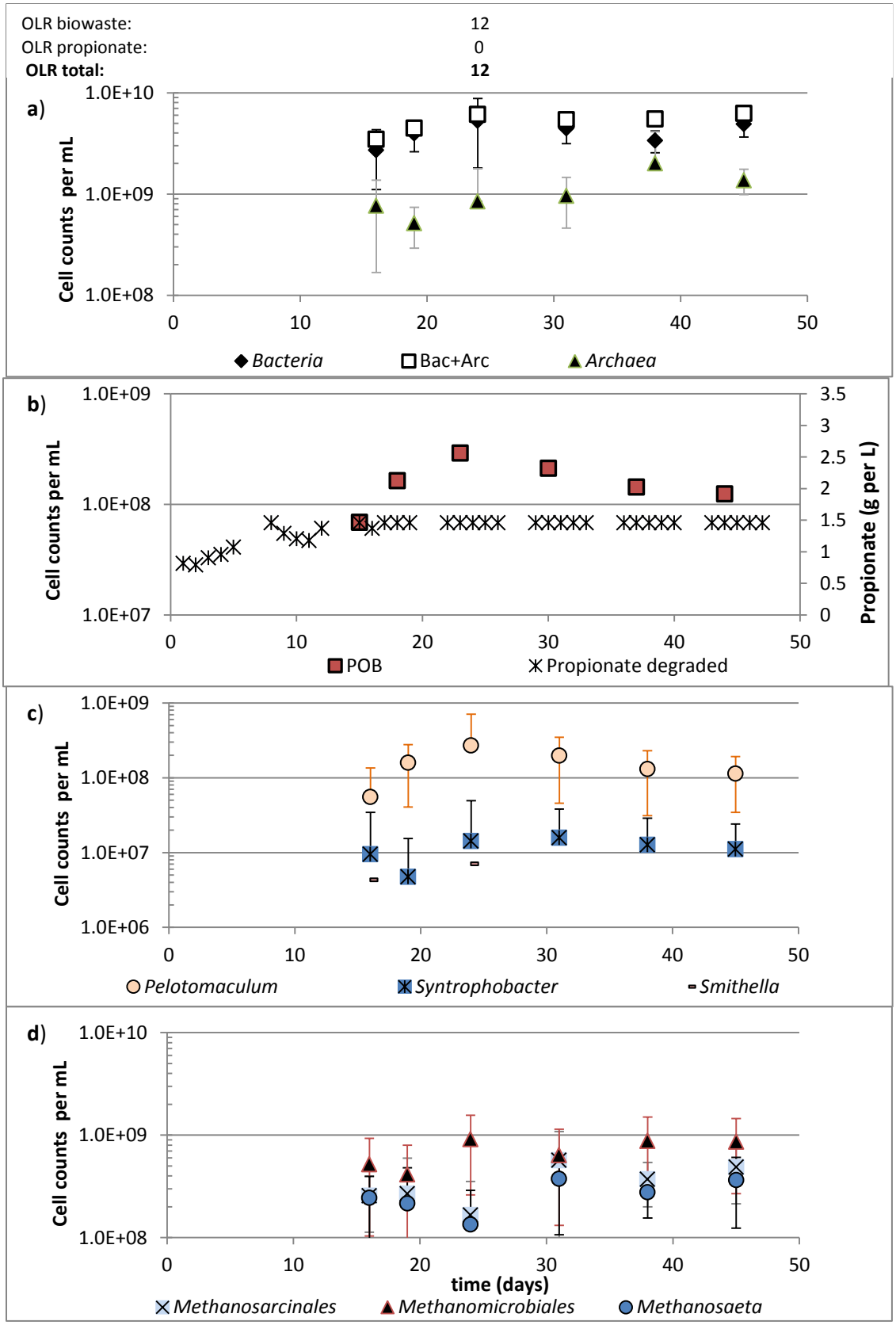

Figure 34: Total *Bacteria* and *Archaea* (a), propionate-oxidizing bacteria (POB) (b, c), and methanogenic *Archaea* (d) in reactor 1, during feeding of biowaste at an OLR of 12.

After 16 days of only biowaste feeding at an OLR of 12 kg COD m^{-3} d^{-1}, the bacterial community consisted of (2 ± 1.1) × 10^9 *Bacteria* and (0.6 ± 0.35) × 10^9 *Archaea* per mL. The addition of propionate was started and numbers of *Bacteria* and *Archaea* increased. The highest numbers of *Bacteria* could be detected 10 days after propionate co-digestion (Li et al. 2015a), (Figure 35a). "Highest cell densities of *Archaea* and highest biogas production were, however, reached about 60 days later" (Li et al. 2015a, Figure 32, Figure 35a),"when up to 2.5 kg $COD_{propionate}$ m^{-3} d^{-1} were supplied in addition to 14 kg $COD_{biowaste}$ m^{-3} d^{-1}, presumably due to much slower growth rates of hydrogenotrophic and/or aceticlastic methanogens than of heterotrophic bacteria" (Li et al. 2015a). Hence "the addition of propionate to the biowaste reactor resulted in a more than 5-fold increase of the numbers of *Archaea* from 0.6 to 3.4 × 10^9 per mL" (Figure 35a). More propionate (up to 4 kg COD m^{-3} d^{-1}) in the presence of 14 kg $COD_{biowaste}$ m^{-3} d^{-1} did not lead to further growth and higher community densities of *Bacteria* and *Archaea*" (Li et al. 2015a)*,* (Figure 35a)". Feeding was interrupted as revision was necessary between day 75 – 85. This had only minor effects on *Bacteria*, but "*Archaea* apparently suffered from starvation and cell numbers decreased more than 50 %, e.g., by cell lysis " (Li et al. 2015a), (Figure 35a). After revision and continued feeding of biowaste + propionate numbers of *Archaea* recovered quickly within 10 days only (Li et al. 2015a).

"When 1 – 1.5 kg $COD_{propionate}$ m^{-3} d^{-1} were fed in addition to 12 kg $COD_{biowaste}$ m^{-3} d^{-1} the community of POB increased more than 3-fold within less than 30 days to at least 5 × 10^8 per mL "(Li et al. 2015a; Figure 35b). The majority of POB were again *Pelotomaculum* sp., whereas the proportion of *Syntrophobacter* and *Smithella* were only 10 and 1 %, respectively (Figure 35c). "During feeding of 1 or 1.5 kg $COD_{propionate}$ m^{-3} d^{-1} and 12 kg $COD_{biowaste}$ m^{-3} d^{-1}, all POB genera increased to their maximum cell density"(Figure 35c). "If more propionate was co-fed the community density of POB did not increase further, but *Smithella* sp. seemed to be less tolerant to high propionate concentrations or were less competitive against upcoming POB and their numbers decreased, similar as during starvation" (Li et al. 2015a, Figure 35c, day 80). One week of starvation during maintenance did not seem to have an effect on *Pelotomaculum* sp. and *Syntrophobacter* sp., and numbers of *Smithella* sp. remained near detection limit. Instead "highest cell numbers for *Pelotomaculum* were (7.1 ± 4.1) × 10^8 per mL and for *Syntrophobacter* (2.2 ± 1.6)× 10^8 per mL" at day 101 (Li et al. 2015a, Figure 35c). Highest cell number of *Smithella* at an earlier time (day 52) were, 3.2 × 10^7 per mL (Figure 35c).

As the addition of propionate was increased from 1 to 2.5 $COD_{propionate}$ m^{-3} d^{-1}, the numbers of *Methanomicrobiales* increased too, parallel as the number of *Archaea* increased (Li et al. 2015a). The maximum cell numbers were (1.8 ± 0.9) × 10^9 per mL at day 73 (Figure 35d). The addition of even more propionate, however, did not lead to higher cell numbers or more growth. When feeding was stopped for maintenance, numbers of *Methanomicrobiales* decreased, whereas numbers of *Methanosarcinales,* including *Methanosaeta,* increased. The gain of *Methanosarcinales* could not overweigh the losses of *Methanomicrobiales*. So the decrease of total *Archaea* was due to the losses of *Methanomicrobiales*. *Methanosarcinales*, among them many cells of *Methanosaeta* sp.*,* were always a minority of methanogens (Li et al. 2015a). Within the first 80 days numbers of *Methanosarcinales* varied between (2.4 ± 1.9)

× 10^8 per mL and (4.9 ± 2.3) × 10^8 per mL, the numbers of *Methanosaeta sp.* between (1.9 ± 1) × 10^8 per mL and (4.7 ± 1.9) × 10^8 per mL (Figure 35d). Cell numbers did not change by the addition of propionate. An increase was determined during revision only and cell numbers remained at a slightly higher level afterwards. Like in reactor 1 *Methanobacteriales* did not play a role, since their number remained low, slightly above the detection limit.

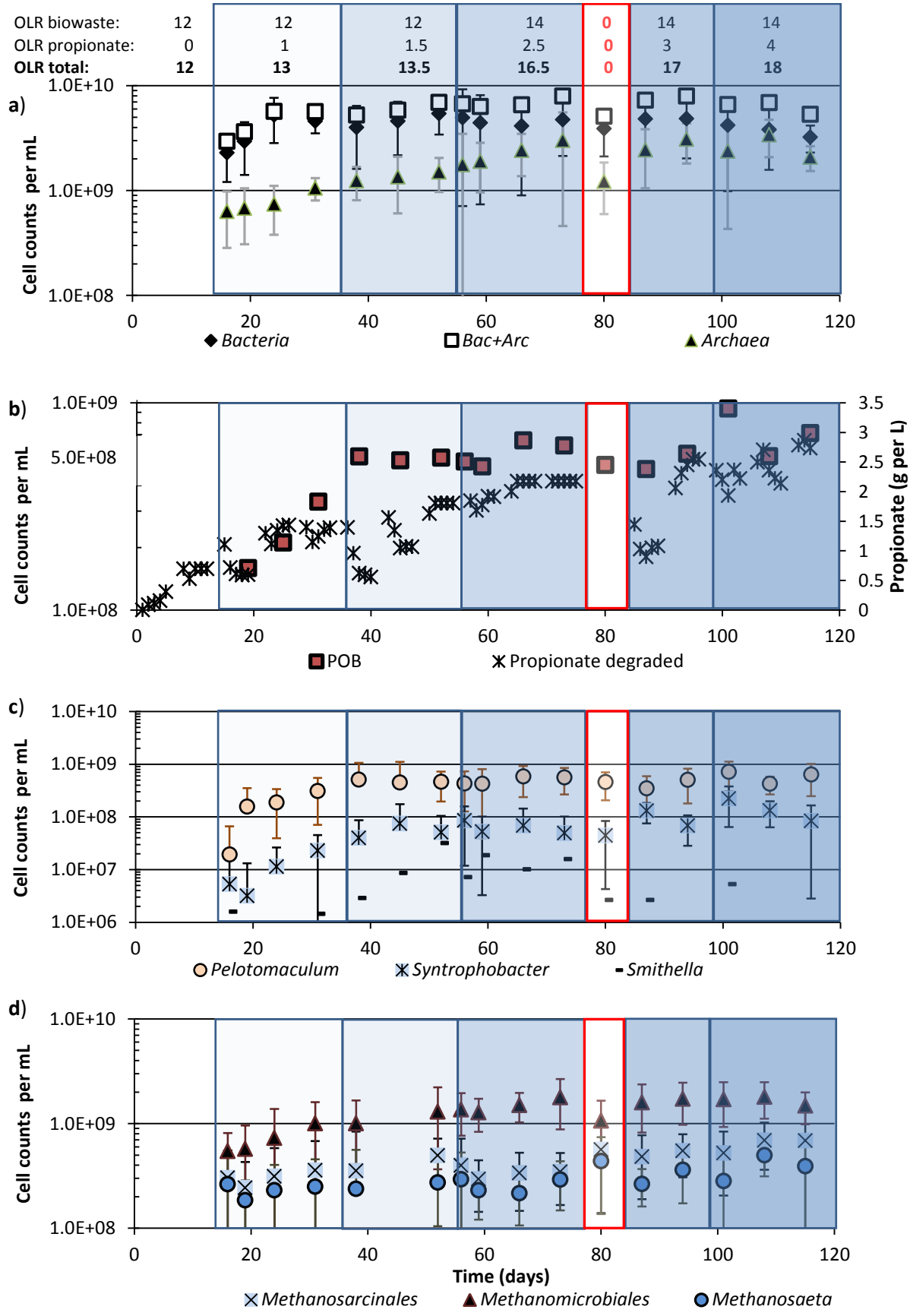

Figure 35: Total *Bacteria* and *Archaea* (a), propionate-oxidizing bacteria (POB) (b, c), and methanogenic *Archaea* (d) in reactor 2, during feeding of biowaste at an OLR of 12 (day 1 – 55) or 14 (day 55 onwards) kg $COD_{biowaste}$ m^{-3} d^{-1} and propionate up to 4 kg $COD_{propionate}$ m^{-3} d^{-1}. Taken from Li et al. (2015a).

In reactor 3, which was fed continuously with propionate and with biowaste 5 days per week numbers of *Bacteria* increased from (2.2 ± 0.7)× 10^9 per mL to (4.7 ± 2.7) × 10^9 per mL (Figure 36a). The numbers of *Archaea* showed a parallel development, as their numbers increased from (0.8 ± 0.5) × 10^9 per mL to (2.8 ± 1.2) × 10^9 per mL . Numbers of the 2 domains slightly decreased after feeding was stopped at day 23 (Figure 36a). Afterwards, when feeding was continued with biowaste, numbers recovered quickly. for the rest of the reactor operation time (days 34-43) both, numbers of *Bacteria* and *Archaea* remained at a stable level, *Bacteria* varying between (2.2 ± 0.6)× 10^9 per mL and (3.2 ± 1.4)× 10^9 per mL and *Archaea* varying between (2.4 ± 1)× 10^9 per mL and (3 ± 1.5)× 10^9 per mL, respectively (Figure 36a). In general there were lower differences between the two domains in reactor 3 than in reactor 1 and 2 (Figure 34a, Figure 35a).

The continuous feeding of surplus propionate led to a fast increase of POB from 1.5 × 10^8 per mL to 9 × 10^8 per mL. Within the first 20 days numbers were at the same level as in reactor 2 after 101 days (Figure 36b). Differences however could be seen between cell numbers obtained directly after weekends and the cell numbers, which originated during the week. After feeding with propionate only during weekends, cell numbers were lower on Mondays than on Wednesdays, when biowaste was fed for already two days, except for the last Monday at day 22, where POB numbers were equally high (Figure 36b). After feeding was stopped cell numbers decreased to 3.8 × 10^8 per mL at day 28, but recovered within 7 days. As the overall amount of added propionate was lower than in the feeding mode between day 6 and 22, cell numbers stabilized at a slightly lower level (5.2 – 5.9) × 10^8 per mL. A weekend effect (lower values on Mondays) could not be determined for this feeding mode. Again, the vast majority of POB belonged to *Pelotomaculum* sp., hence numbers of total POB and of *Pelotomaculum* sp. showed a parallel development, (1.4 ± 0.8) × 10^8 per mL to (8.2 ± 4) × 10^8 per mL within the first 22 days (Figure 36c). There were also increasing numbers of *Syntrophobacter* sp.. Like in the case of POB and *Pelotomaculum* sp., less cells were detected on Mondays. After the interruption of feeding both, numbers of *Syntrophobacter* sp. and of *Pelotomaculum* sp. increased within 7 days and remained stable for the rest of the operation time (Figure 36c). Although *Smithella* sp. could be detected, cell numbers these species remained stable at a low level and did not increase above the level of 1 × 10^7 per mL. Only on day 8 1.4 × 10^7 per mL were counted (Figure 36c).

Analyses of methanogenic subgroups revealed, that *Methanomicrobiales* and *Methanosarcinales* were nearly equally represented. Numbers of *Methanomicrobiales* increased from (0.4 ± 0.3) × 10^9 per mL to (1.4 ± 0.5) × 10^9 per mL and number of *Methanosarcinales* from (0.5 ± 0.2) × 10^9 per mL to (1.6 ± 0.9) × 10^9 per mL (Figure 36d). The subgroup of *Methanosarcinales* contained itself high numbers of *Methanosaeta*. Their cell numbers increased from (3.2 ± 1.8) × 10^8 per mL to (8.7 ± 5.2) × 10^8 per mL (Figure 36d). After feeding was stopped, cell numbers declined: The numbers of *Methanomicrobiales* to (0.7 ± 0.4) × 10^9 per mL and the numbers of *Methanosarcinales* to (0.4 ± 0.2) × 10^9 per mL. The cell counts of *Methanomicrobiales* recovered more quickly to (1.5 ± 0.7)× 10^9 per mL and remained at this level for the rest of the operation time. Numbers of *Methanosarcinales*

did not increase within the first days. This was due to *Methanosaeta* sp., whose cell counts decreased even further after the first weekend with a feeding of 5 kg $COD_{propionate}$ m^{-3} d^{-1} to (2.4 ± 1.2) × 10^8 per mL. In the following days numbers began to increase, however, till the cell counts of *Methanosarcinales* finally reached the level of the *Methanomicrobiales.* Hence their proportion returned to the quota of the first day of operation (Figure 36d).

The comparison of percentages of POB in reactor 1 and reactor 2 revealed, that not only the absolute numbers were higher, but also the relative proportions (Figure 34, Figure 35, Figure 37a). The first strong increase of *Syntrop*hobacter sp. within the proportion of POB was at the time, when feeding of propionate was increased from 1 to 1.5 kg $COD_{propionate}$ m^{-3} d^{-1} (Figure 37a). At a feeding of 1.5 and 2.5 kg $COD_{propionate}$ m^{-3} d^{-1} the numbers and the proportions of *Pelotomaculum* sp. and *Syntrophobacter* sp. remained about the same. The increase of the OLR by propionate to 2.5 kg did not change the percentages of POB within the total population. After interruption and re-starting feeding with biowaste and a high load of propionate, the percentage of POB remained around 10 %. At the highest propionate load of 4 kg COD m^{-3} d^{-1} OLR an increase of the POB percentage could be seen: More than 20 % of all *Bacteria* belonged to POB taxa. At this time the proportion of *Syntrophobacter* sp. within the POB was the highest of all samples collected during propionate co-digestion experiments. Up to 2.6 % and 5.2 % of *Bacteria* belonged to *Syntrophobacter* sp. (Figure 37a). In reactor 3 the relative proportion of POB was also higher than in reactor 1 (Figure 37b). When biowaste and propionate was fed (day 8 and 15), the proportion of POB within the Bacteria was around 10 %. The numbers originated from Mondays, which represented feeding with only propionate during weekends. A strong increase was seen at day 20 and 22, when the proportion of POB was above 25 %. When feeding was continued, the proportion of POB sank from 21 % to 12 % within the first week, but recovered and reached similar proportions as on day 20 and 22 later on (Figure 37b).

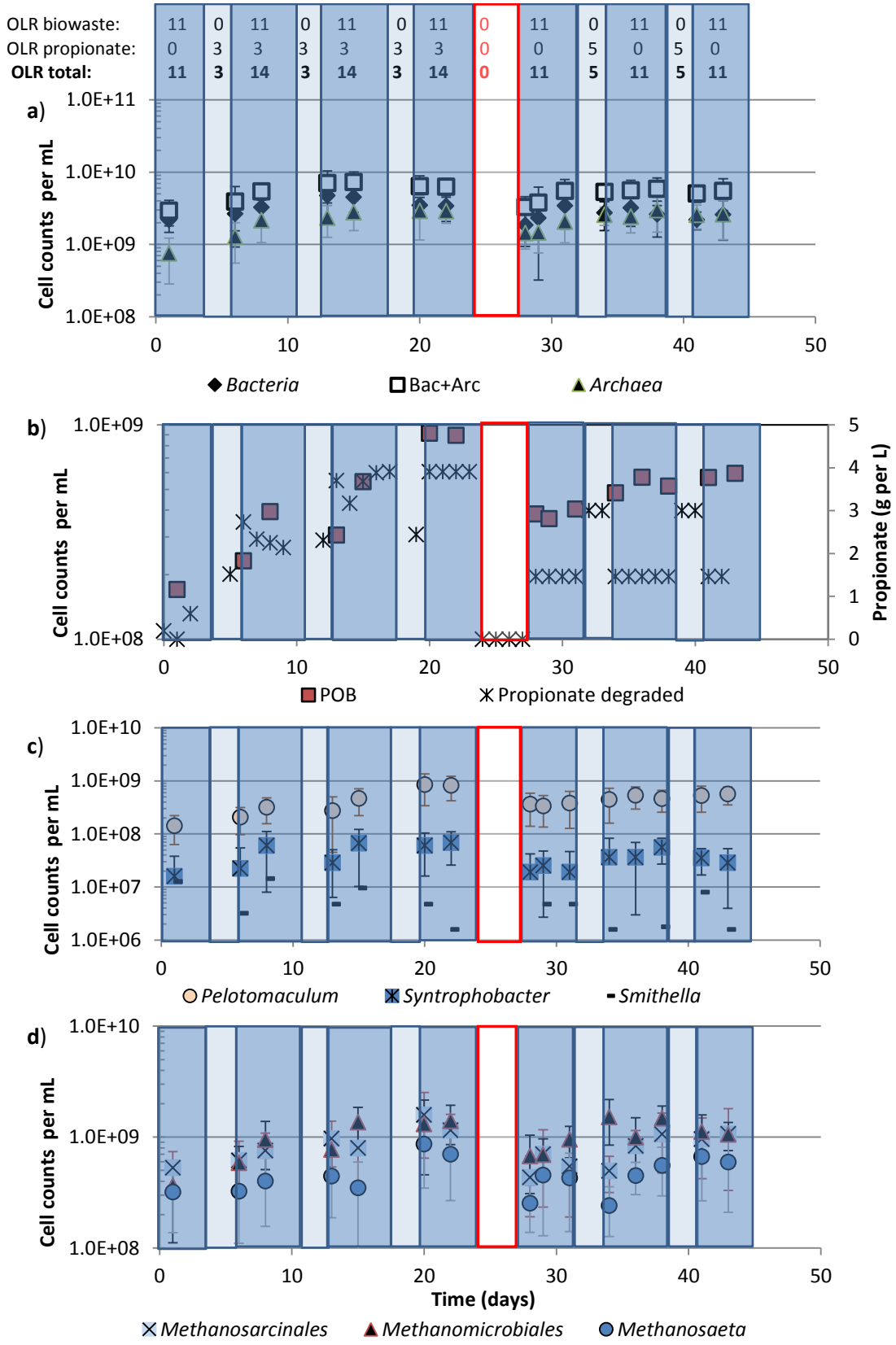

Figure 36: Total *Bacteria* and *Archaea* (a), propionate-oxidizing bacteria (POB; b, c), and methanogenic *Archaea* (d) in reactor 3, during continuous feeding of propionate at an OLR of 3 kg $COD_{propionate}$ m^{-3} d^{-1} and biowaste at an OLR of 11 kg $COD_{biowaste}$ m^{-3} d^{-1} till day 22. From day 28 biowaste (11 kg $COD_{biowaste}$ m^{-3} d^{-1}) was fed Monday till Friday and propionate (5 kg $COD_{propionate}$ m^{-3} d^{-1}) from Friday till Monday.

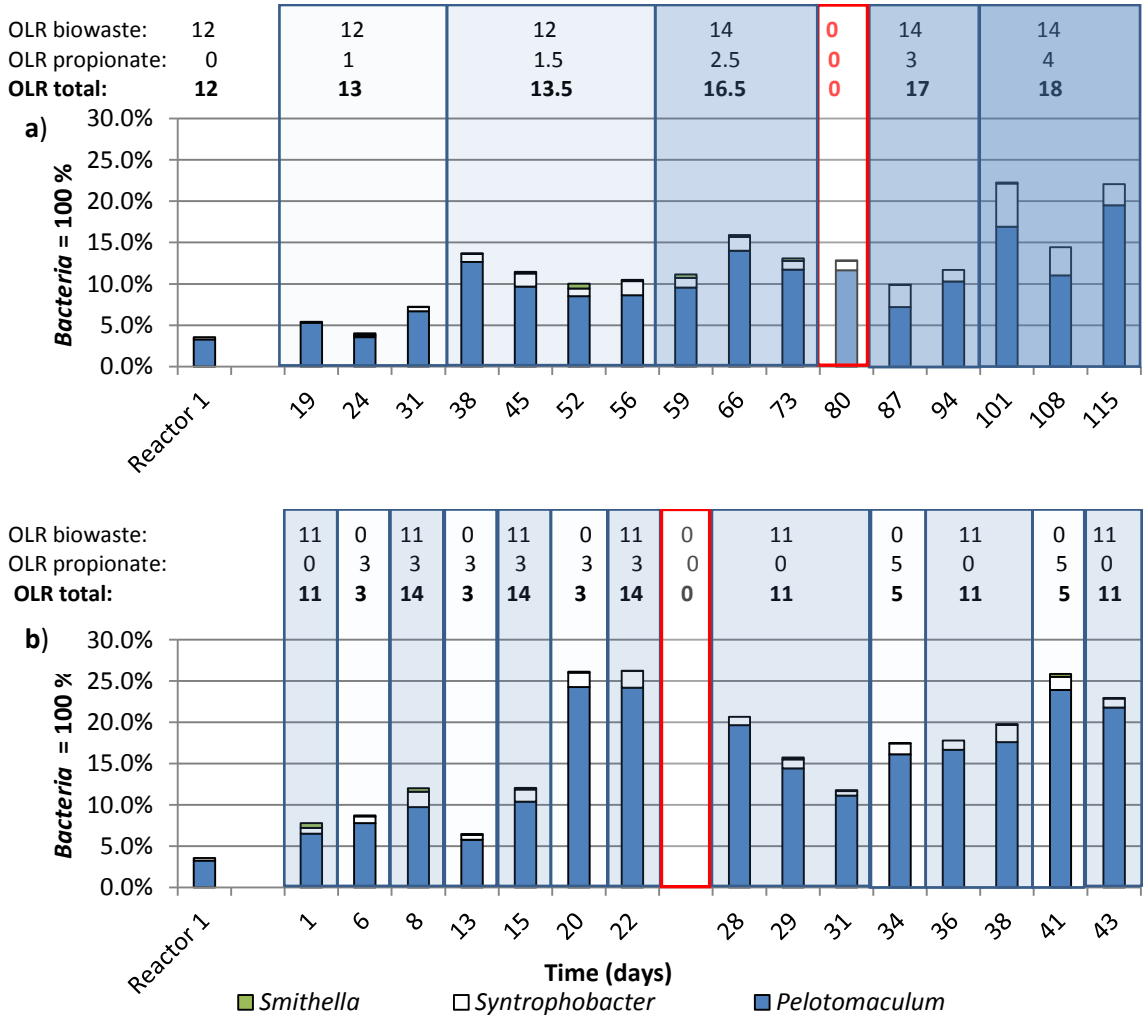

Figure 37: Proportions of POB taxa in reactor 2 (a) and reactor 3 (b). Samples taken on Mondays (days, 6, 13, 15, 34, 41) were accounted to feeding mode of weekends. An average value for reactor 1 was calculated by using the mean of the three last sample times (31, 38, 45 days). OLR values in kg COD m^{-3} d^{-1}. Adapted from Li et al. (2015a).

If the methanogenic subtaxa in reactor 2 and reactor 3 were compared, it could be seen, that the proportions of *Methanomicrobiales* were higher and comprised almost two thirds of all methanogens (Figure 38a). On the other hand a decline of *Methanosaeta* with higher propionate addition could be seen in reactor 2. Whereas proportions of *Methanosaeta* were between 18 % and 23 % for a propionate addition of 1 kg $COD_{propionate}$ m^{-3} d^{-1}, only 13 – 16 % of methanogens were *Methanosaeta* at 2.5 kg $COD_{propionate}$ m^{-3} d^{-1} (Figure 38a). In reactor 3 the numbers of "other *Methanosarcinales*" species, which belong to this order but not to *Methanosaeta* (e.g. *Methanosarcina* sp.), were higher, especially between day 1 and 22, when their proportion reached the level of 20 % of all methanogens (Figure 38b). After re-starting the feeding at day 28, proportions of *Methanomicrobiales* were slightly higher, always near or above 50 % (Figure 38b).

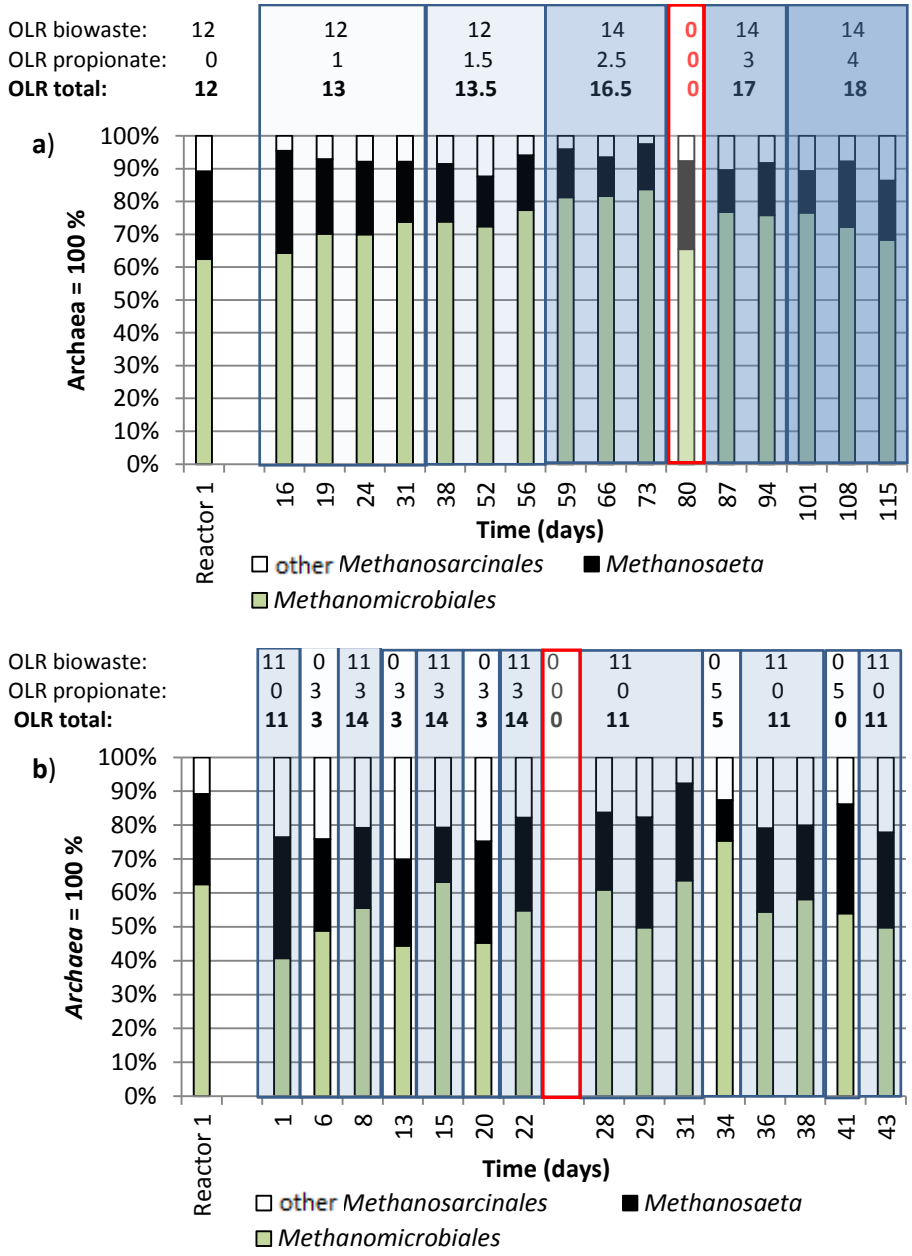

Figure 38: Proportions of Methanogens in reactor2 (a) and reactor 3 (b) Adapted from Li et al. (2014). Samples taken from Mondays (days, 6, 13, 15, 34, 41) were accounted to feeding mode of weekends. An average value for reactor 1 was calculated by using the mean of the three last sample times (31, 38, 45 days). OLR values in kg COD m^{-3} d^{-1}. Adapted from Li et al. (2015a).

3.5 Mesophilic and thermophilic dry anaerobic digestion

Samples from the dry anaerobic digestion (DAD) reactors that contained biowaste with 30 % DM content in general contained less bacteria than those of DAD reactors containing biowaste with a higher moisture content, as these reactors produced only small amounts of biogas. The biowaste acidified by formation of high concentrations of VFA, which remained throughout 60 days of incubation, reaching final concentrations of 25 and 31 g VFAs per L (Figure 39). In the reactors that contained biowaste with 25 or 20 % DM VFAs were produced and degraded within 60 d, accompanied by respective biogas production. However, some propionate, between 0.2 g and 10 g per L, remained un-degraded in these DAD reactors (Figure 39). As already presented by Li et al. (2014a) the cell numbers of total prokaryotes, including *Bacteria* and *Archaea,* depended on the dry matter content of the DAD reactors, whereas temperature had not a major effect on cell numbers. There were no clear developments towards higher or lower cell numbers within 52 days of incubation. DAPI stainable cell numbers were between (2.5 ± 0.85) x 10^9 to (7.5 ± 3.55) x 10^9 cells per ml, cell numbers of *Bacteria* between (0.5 ± 0.3) x 10^9 to (2.7 ± 1.6) x 10^9 cells per ml. There was however a clear difference between *Archaea* cell numbers in the DAD reactors that contained 20 and 25 % DM on the one hand and the cell numbers in the DAD reactor, that contained 30 % DM. Archaeal cell numbers in the DAD reactors that contained 20 and 25 % DM were (8.3 ± 5.8) x 10^8 to (9.4 ± 4.7) x 10^8 per ml and thus twice as high as the archaeal population in the DAD reactor with 30 % DM, where cell numbers of (2.4 ± 1.9) x 10^8 at 55°C and (3.4 ± 2.4) x 10^8 per g at 37°C were determined (Table 13, Figure 40) methanogenic orders were used, namely probes specific for *Methanosarcinales*, *Methanosaeta*, *Methanobacteriales* and *Methanomicrobiales* (Raskin et al. 1994). The highest numbers were determined for the order of the *Methanosarcinales*, which contributed up to 96 % to the methanogenic community at both temperatures, 37 or 55 ^oC (Figure 41). A few percent of the methanogens belonged to the *Methanomicrobiales*. Only the samples from the DAD reactors that contained biowaste with 30 % DM content for digestion revealed a higher diversity of methanogens, as *Methanomicrobiales* contributed almost 20 % of the archaeal community and *Methanobacteriales* were also detected (Figure 41). *Methanosaeta* related organisms could not be found in all DAD reactor samples, thus it can be assumed that their number is below 1.6 x 10^6 cells per mL.

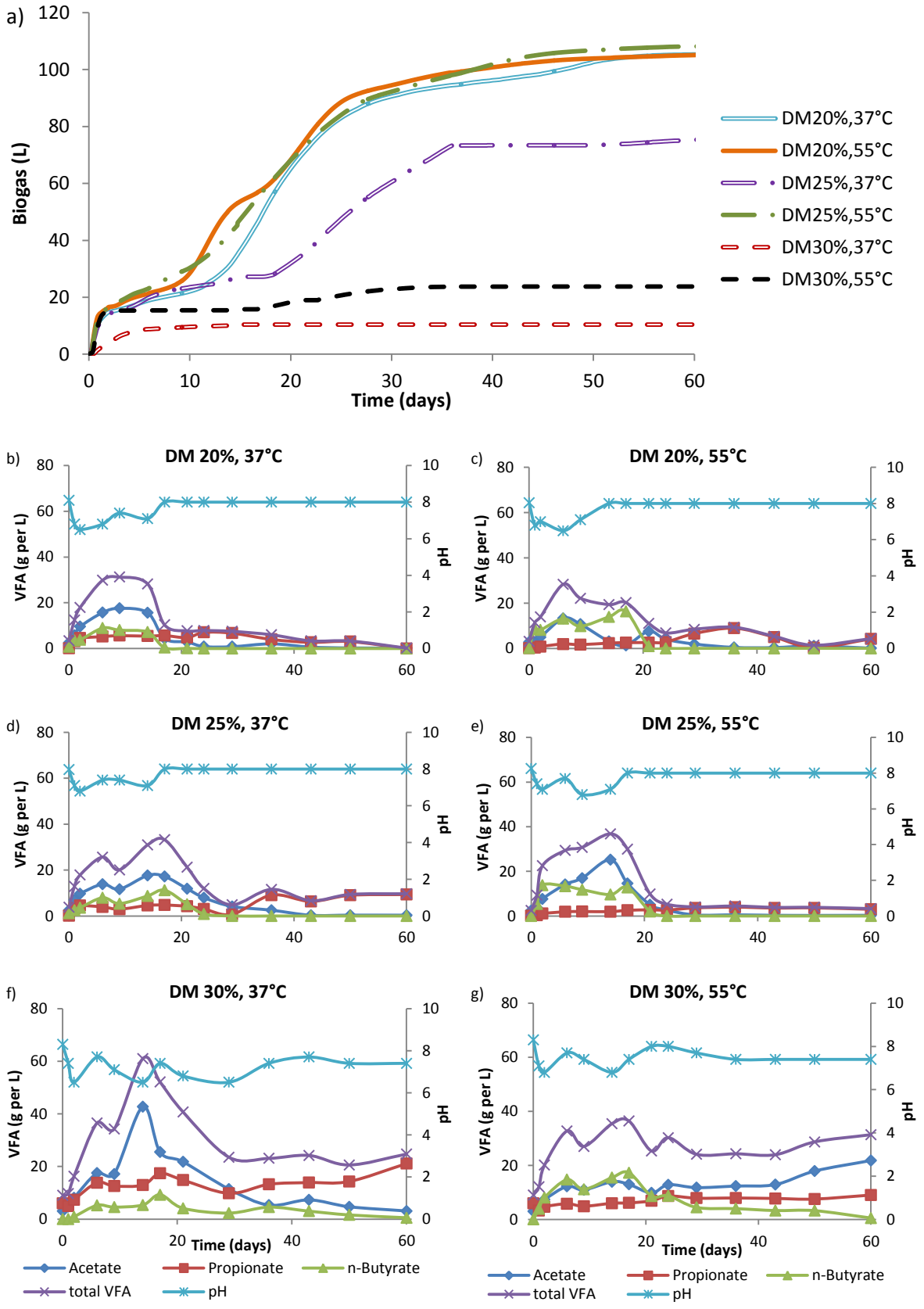

Figure 39: Biogas production in biowaste reactors with 20, 25 and 30 % DM content (a), pH and volatile fatty acid (VFA) concentrations after re-feeding the reactors at mesophilic (37 °C; b,d,f) or thermophilic (55 °C; c,e,g) temperatures. Taken from Li et al. (2014a).

Table 13: Counted cells per g of biowaste from the DAD reactors with 30, 25 and 20 % dry matter content after staining with DAPI and fluorescence marking with the gene probes Eub388 and Arc915.

	Time(days)	DAPI x10^9		Eub388 x10^9		Arc915 x10^9	
		mean	SD	mean	SD	mean	SD
DM30%,t	21	2.5	0.9	0.5	0.3	0.2	0.2
DM30%,t	36	3.8	1.1	1.0	0.5	0.2	0.1
DM30%,t	52	3.6	2.0	1.4	1,2	0.5	0.4
DM30%,t	Total counts[1]	3.0	1.2	1.0	0.5	0.4	0.3
DM30%,m	21	3.0	1.3	0.9	0.5	0.3	0.2
DM30%,m	36	4.3	1.8	1.2	0.5	0.5	0.3
DM30%,m	52	1.8	0.4	0.9	0.5	0.4	0.4
DM30%,m	Total counts[1]	3.4	1.3	1.0	0.7	0.3	0.2
DM25%,t	21	4.1	1.4	1.1	0.4	0.8	0.5
DM25%,t	36	4.0	1.1	1.0	0.5	0.6	0.4
DM25%,t	52	3.9	1.7	1.0	0.5	0.5	0.3
DM25%,t	Total counts[1]	4.2	1.4	1.3	0.9	0.8	0.3
DM25%,m	21	6.0	1.7	1.5	0.7	0.9	0.2
DM25%,m	36	3.0	1.1	0.7	0.9	0.5	0.3
DM25%,m	52	3.7	1.3	1.8	0.9	0.9	0.3
DM25%,m	Total counts[1]	4.0	3.3	1.1	0.5	0.6	0.4
DM20%,t	21	7.5	3.6	2.7	1.6	1.0	0.5
DM20%,t	36	3.5	1.1	1.4	0.7	0.6	0.4
DM20%,t	52	4.2	0.7	1.5	0.5	0.5	0.3
DM20%,t	Total counts[1]	4.4	1.2	1.4	0.8	0.7	0.3
DM20%,m	21	4.8	1.2	1.7	1.0	0.9	0.4
DM20%,m	36	5.1	1.7	1.5	1.0	0.6	0.3
DM20%,m	52	3.3	0.7	0.8	0.5	0.6	0.4
DM20%,m	Total counts[1]	5.0	1.8	1.9	0.9	0.7	0.4

[1]Mean and standard deviation (SD) of the 3 analyses per sample (n = 10) 21, 36 and 52 days after start of the reactors.

POB were found in all samples (Figure 42). In general more POB were detected in the samples of the DAD reactor that contained biowaste with 20 % DM content. It could also be seen that the numbers in the mesophilic assays were higher than in the thermophilic assays. The proportion of POB to *Bacteria* was between 0.4 – 0.8 % in the DAD reactors containing biowaste with 30 % DM content and 5 – 6 % in the reactor containing biowaste with 20 % DM content. As there are different organisms in mesophilic and thermophilic assays

different FISH probes had to be used. In the thermophilic reactor with 30 % DM containing biowaste the percentage of *Desulfotomaculum thermobenzoicum thermosyntrophicum* was the lowest, instead a large proportion of mesophilic *Syntrophobacter* sp. were found. By time the proportions changed to a majority of *Desulfotomaculum thermobenzoicum thermosyntrophicum* (Figure 43). In the thermophilic reactor with 25 % DM containing biowaste just the opposite was the case: at day 21 two third of the POB population belonged to *Desulfotomaculum*, but at day 52 only 40 % POB remained. In the assay with 80 % moisture (20 % DM content of biowaste) a vast majority of *Desulfotomaculum* was determined within the whole incubation time (Figure 43). In the mesophilic reactor with 25 % DM containing biowaste "the percentage of *Pelotomaculum* sp. was decreasing with time whereas the percentage of *Syntrophobacter* sp. increased as long as propionate was available, and then decreased" (Li et al. 2014a). In the mesophilic samples *Pelotomaculum* sp. and *Syntrophobacter* sp. were found, whereas *Smithella* sp. could not be found (Figure 44). The proportion of *Pelotomaculum* sp. increased strongly in the reactor with 20 % DM containing biowaste (Figure 44).

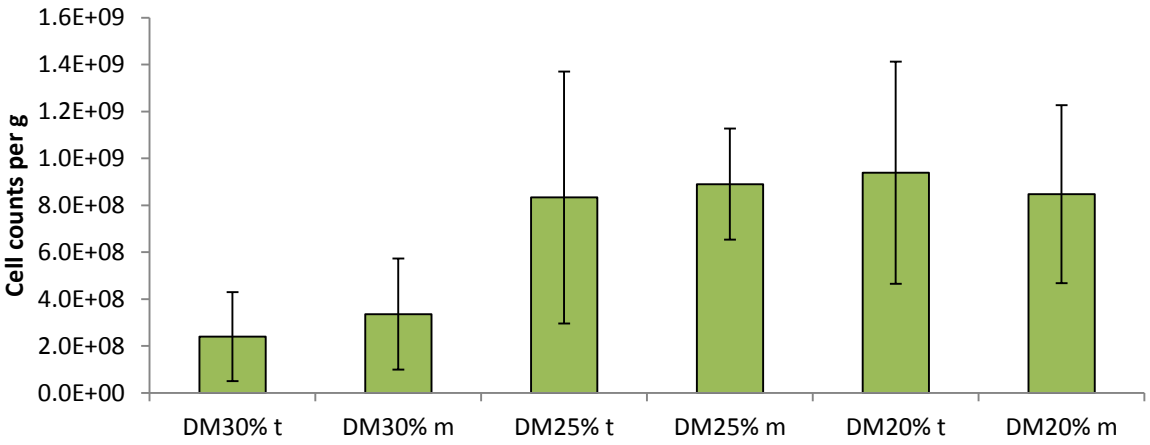

Figure 40: Mean numbers and standard deviation of *Archaea* per g sample (n=10). Modified from Li et al. (2014).

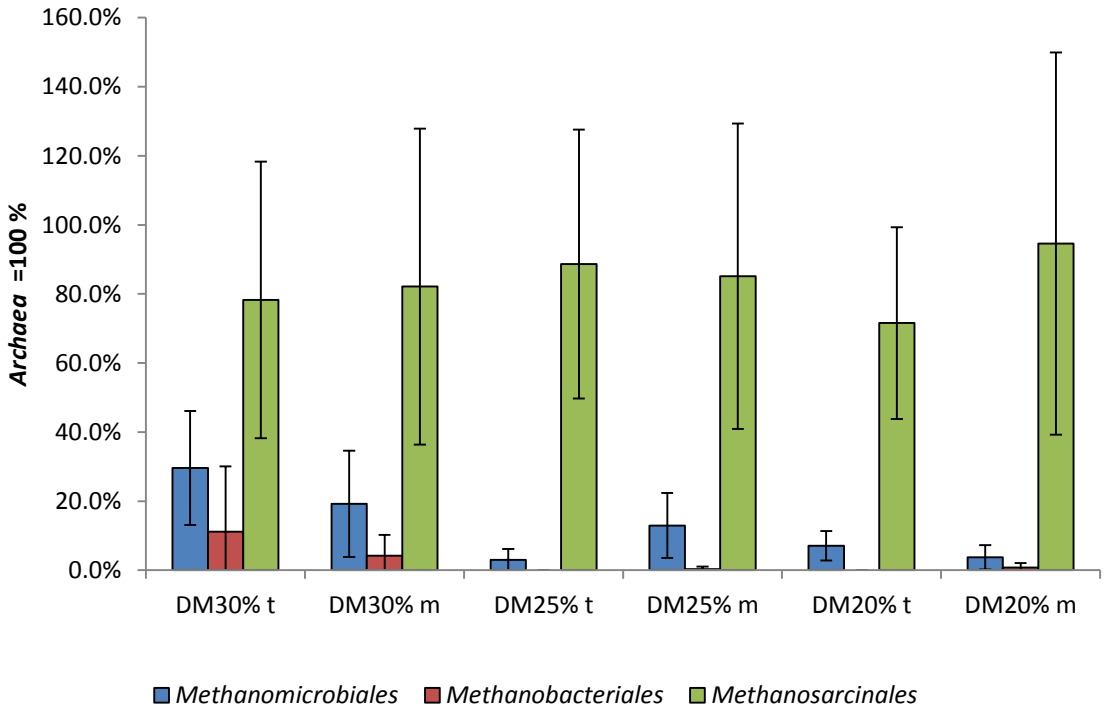

Figure 41: The proportion of major methanogenic taxa (% *Methanosarcinales*, *Methanobacteriales* and *Methanomicrobiales*) in DAD reactors with biowaste that contained 30, 25, and 20 % DM at mesophilic (m, 37°C) and thermophilic (t, 55°C) temperature after 21 days. Taken from Li et al. (2014).

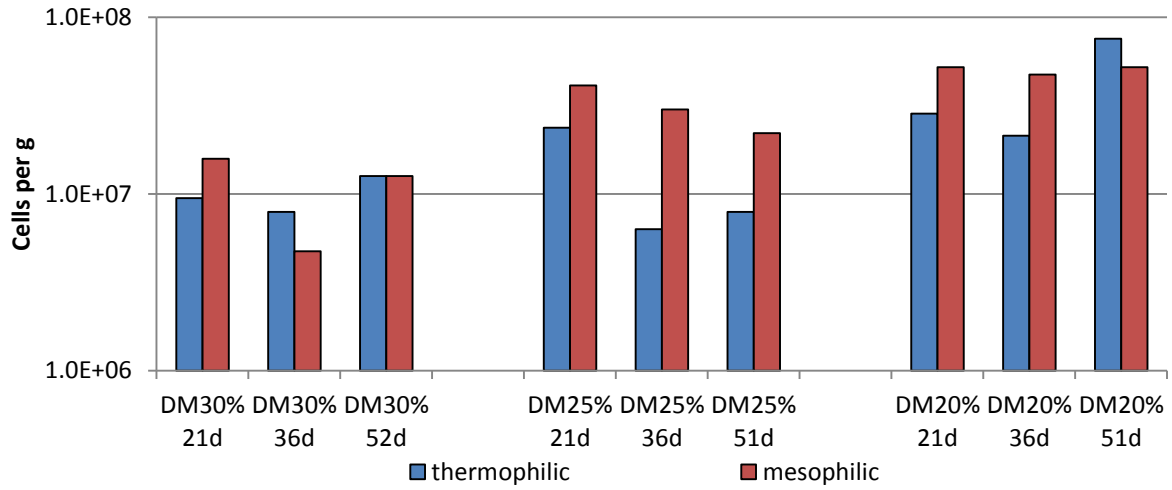

Figure 42: Mean numbers of POB counted in different samples (n=10).

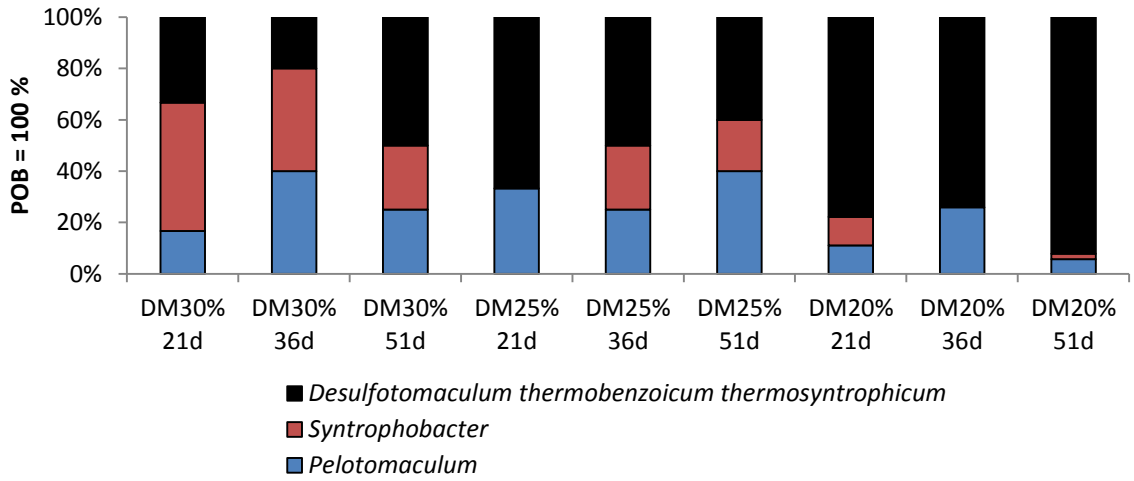

Figure 43: Proportion of POB taxa in the thermophilic reactor (55 °C).

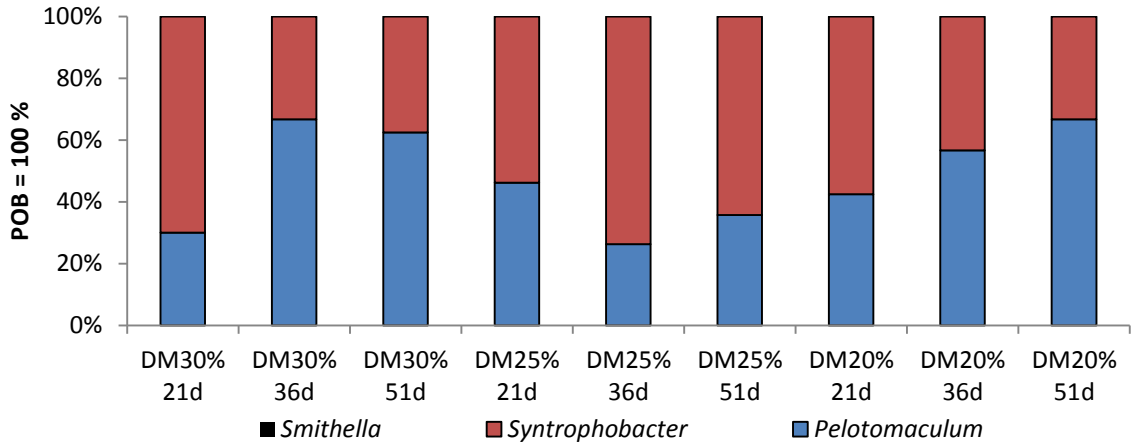

Figure 44: Proportion of POB taxa in the mesophilic reactor (37°C). Adapted from Li et al. (2014a).

3.6 Probe Evaluation for detection of Desulfotomaculum thermobenzoicum thermosyntrophicum

The properties of probe Dtsyn1130 for detection of *Desulfotomaculum thermobenzoicum thermosyntrophicum* via fluorescence in situ hybridization are summarized in Table 14. It could clearly be seen that the probe fulfills all the requirements mentioned in Table 10. The possible homo-dimers should not play any role, as the binding energy is low, and hence the oligonucleotide should be single stranded at a FISH hybridization temperature of 46°C, which is required for optimal binding at the target ribosome subunit (Figure 45). If the sequence of probe Dtsyn1130 is compared with the 16s rRNA gene sequence of the target organism and several non-target organism, it could be seen that the relevant sequence changed by insertion of an adenine base and by exchange of a cytosine by adenine (Figure 46). The insertion of adenine is responsible that only the first 9 bases of probe Dtsyn1170 can match

with non-target organisms. Although many organisms with different relatedness were tested, the relevant sequence was the same in all non-target organisms (Figure 46).

Table 14: Characteristics of probe Dtsyn1130. Data were obtained by using Oligo Analyzer software.

Probe properties	Dtsyn1130
Sequence	GGT TAA GTC ACA GCA ACG AGC G
Length	22 bases
GC content	54,5%
T_m(basic)	68.0°C
T_m (salt concentration 50mM)	64.0°C
T_m (nucleotide concentration 200 pM)	66.4°C
Molecular weight	6855.4 g per mol
ΔG (energy required to dissolve a perfect match double strand)	-42,6 kcal per mol

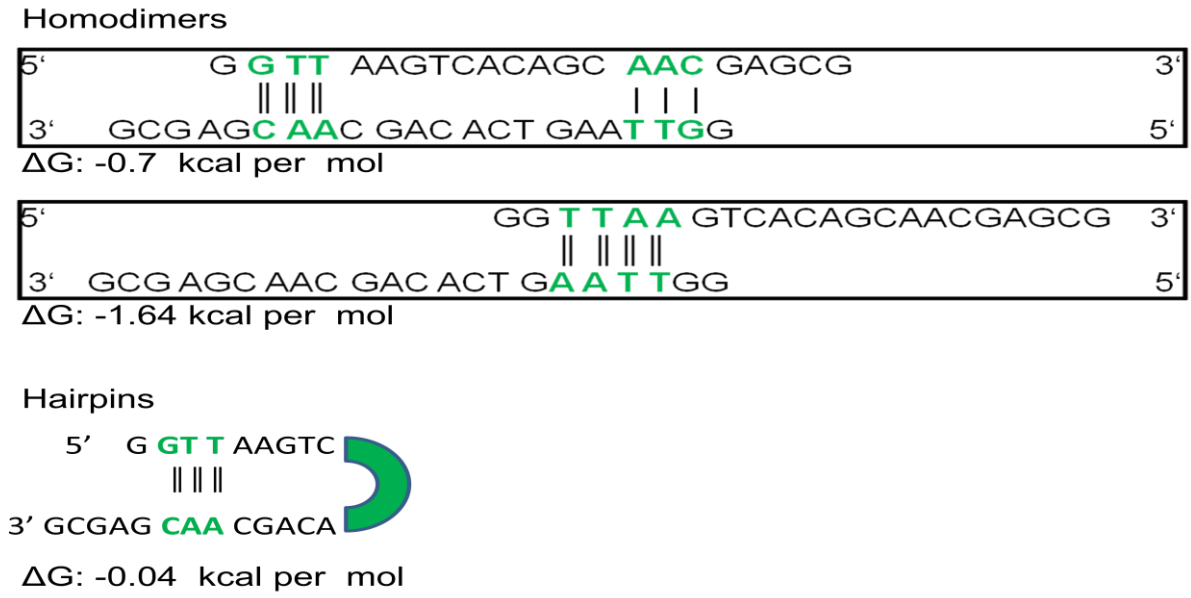

Figure 45: Possible homo-dimers and hairpins of probe Dtsyn1130 and the energy required to dissolve them (calculated using Oligo Analyzer software).

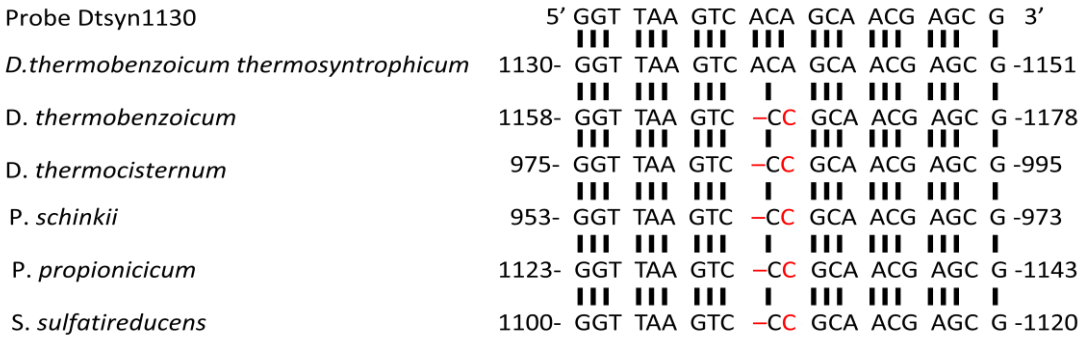

Figure 46: Sequence of probe Dtsyn1130 and analogue sequences of the 16s rRNA gene sequence.

After all probe properties were tested in silico, FISH experiments with the target organism *Desulfotomaculum thermobenzoicum thermosyntrophicum* were performed using a broad range of formamide concentrations. The same broad range of formamide concentrations was applied to *Desulfotomaculum thermobenzoicum thermobenzoicum*. As can be seen in Figures 47a, b, e and f the two subspecies are not easily distinguishable, either by non-selective staining or by phase contrast microscopy. The FISH experiments revealed, that probe Dtsyn1130 produced brightness intensities above 20 RU at 10 and 20 % formamide (Figure 48). At 25 % formamide there was a decrease in brightness to 15.7 RU. At even higher formamide concentrations RU values were lower than 10 RU. When the closest related non-target organism was tested, it was observed that at 10 % formamide there were no relevant differences between target and non-target cells, as 19.7 RU were obtained by *Desulfotomaculum thermobenzoicum thermobenzoicum*. However, brightness intensity decreased even at 20 % formamide content to 15.7 RU and at 25 % formamide to 10.3 RU (Figure 48). At the higher formamide concentrations (30 – 60 %) brightness intensity was lower than 10 RU. So the major differences in brightness intensity were seen at 20 % and at 25 % formamide, with difference values of 5.2 and 5.4 RU, respectively (Figure 48).

At a formamide concentration of 10 % most of the more distantly related tested organisms revealed equal brightness intensity like the target organism of around 20 RU. Even the cells of the most distantly related organism *Syntrophobacter sulfatireducens* had an intensity of 18.1 RU (Figure 49). The only exception was *Desulfotomaculum thermocisternum*, whose cells revealed a lower brightness at every formamide concentration (Figure 49). At a formamide concentration of 20 % *Pelotomaculum schinkii* revealed an almost similar strong brightness of 14 RU as *Desulfotomaculum thermobenzoicum thermobenzoicum*. The other non-target organisms did not have brightness higher than 10 RU. At 30 % formamide the fluorescence of all non-target organisms was below 10 RU and thus at the same low brightness as the target organism (Figure 49).

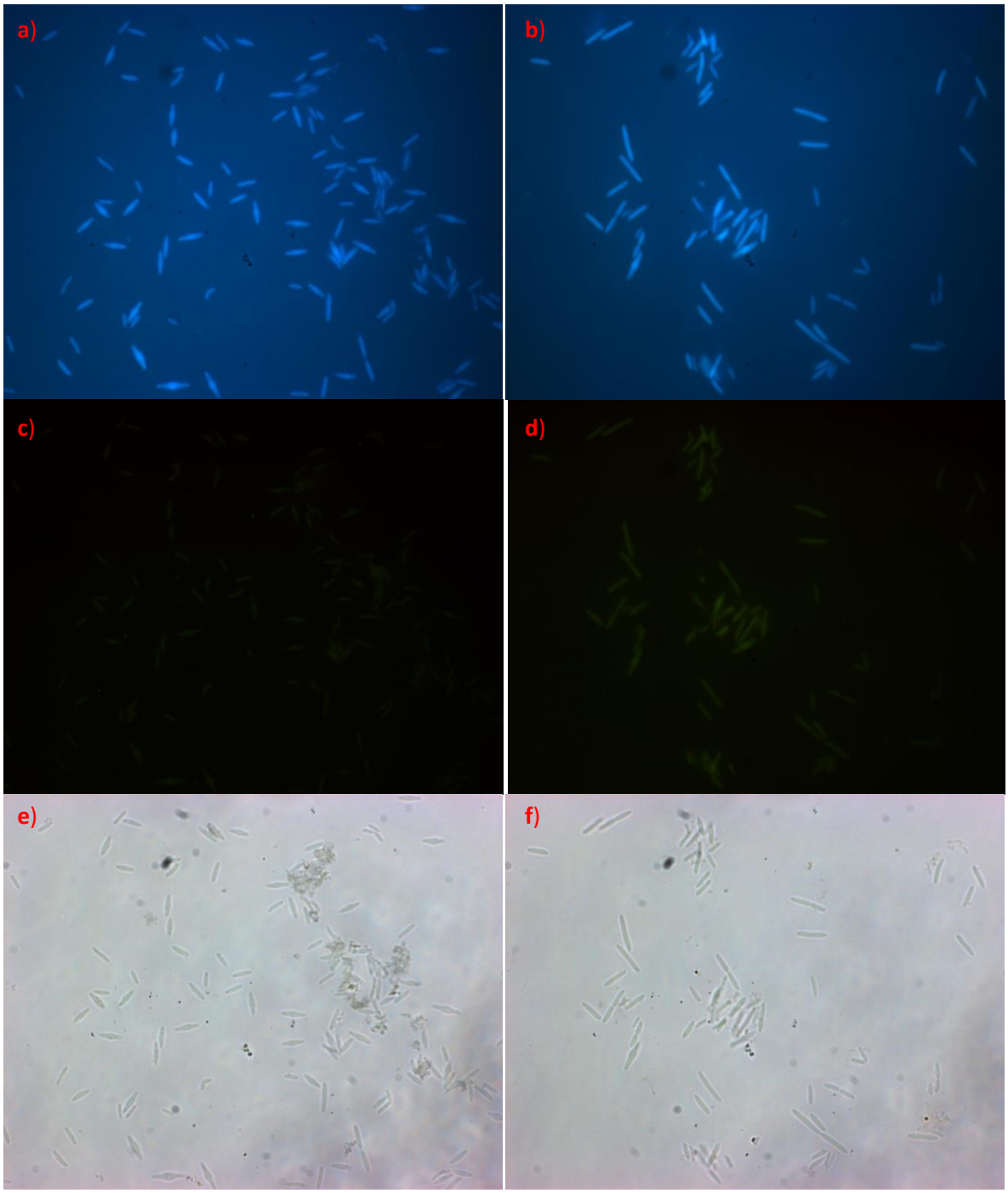

Figure 47: Images of *Desulfotomaculum thermobenzoicum thermobenzoicum* (a, c, e) and *Desulfotomaculum thermobenzoicum thermosyntrophicum* (b, d, f). DAPI (a,b) and FISH using probe Dtsyn1130 at 20 % formamide with an exposure time of 30000 ms (c, d).

A positive control using probe Eub388 was performed with all organisms, which were subjected to probe Dtsyn1130. Results showed that a fluorescence value around 20 RU should be obtained. The highest brightness was calculated from the images of *Desulfotomaculum thermobenzoicum thermosyntrophicum* followed by *Pelotomaculum schinkii* with slightly lower brightness intensity (Figure 50). *Desulfotomaculum thermobenzoicum thermobenzoicum*, *Desulfotomaculum thermocisternum* were little above,

whereas *Pelotomaculum propionicicum* and *Syntrophobacter sulfatireducens* were slightly below 20 RU (Figure 50).

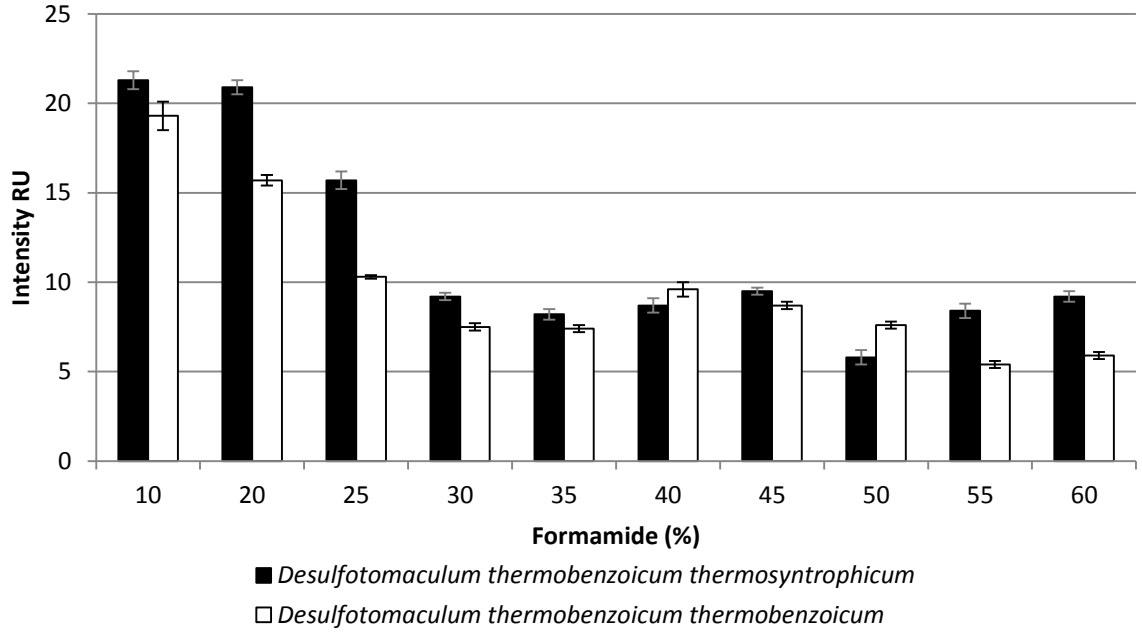

Figure 48: Brightness intensity of probe Dtsyn1130 at different formamide concentrations applied for detection of the target organism *Desulfotomaculum thermobenzoicum thermosyntrophicum* and the closest related organism *Desulfotmaculum thermobenzoicum thermobenzoicum*.

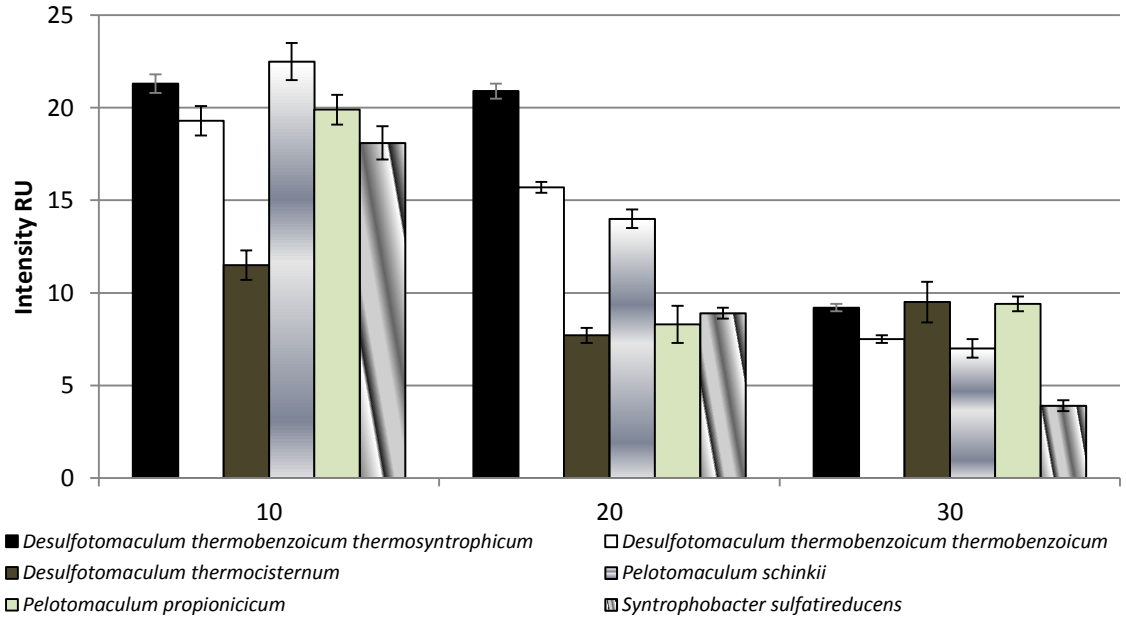

Figure 49: Brightness intensity of Probe Dtsyn1130 at 10, 20 and 30 % formamide applied for detection of the target organism *Desulfotomaculum thermobenzoicum thermosyntrophicum* and various closely and distantly related non target species.

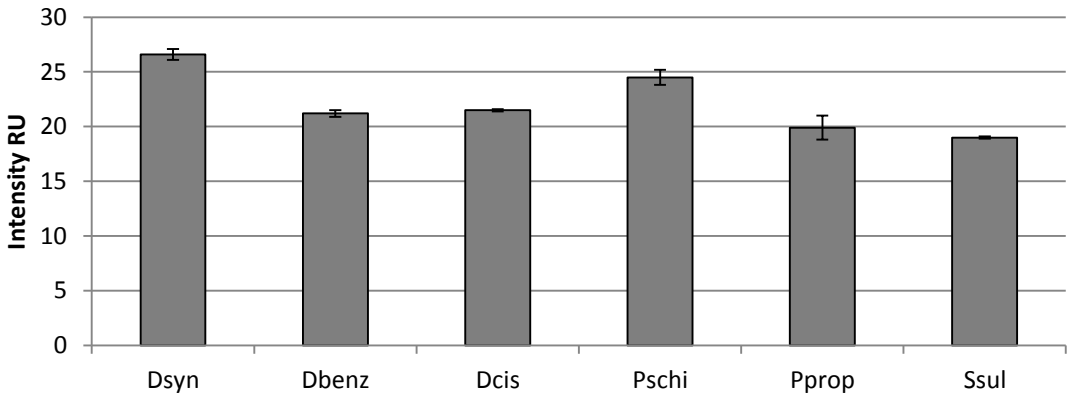

Figure 50: Brightness intensity of probe Eub388 (positive control) at 30 % formamide applied for detection of various bacterial species (Dsyn: *Desulfotomaculum thermobenzoicum thermosyntrophicum,* Dbenz: *Desulfotomaculum thermobenzoicum thermobenzoicum*, Dcis: *Desulfotomaculum thermocisternum*, Pschi: *Pelotomaculum schinkii*, Pprop: *Pelotomaculum propionicicum*, Ssul *Syntrophobacter sulfatireducens*).

4. Discussion

4.1 Enrichment cultures and co-cultures consisting of two single species

In the work of Felchner-Zwirello (2013), who used the same enrichment culture, the degradation of propionate was even at a higher level, between 5 and 9 days for the degradation of 10 mM propionate, hence the degradation rate was between 1.1 and 2 mM per d. Although it is known that most POB grow faster when associated with a syntrophic partners than in axenic cultures (Table 3), growth rates and propionate degradation rates as well were unusually high compared to most of the other *Syntrophobacter* sp., except for *Syntrophobacter sulfatireducens,* which seems to be the closest relative to the *Syntrophobacter* like organism in the mesophilic propionate-oxidizing bi-culture. In the study of Wallrabenstein et al. (1995), it took 16 days to degrade 8 mM propionate by a pure culture of *Syntrophobacter pfennigii* (degradation rate 0.5 mM per d). There is also another work using *Syntrophobacter fumaroxidans* in a pure culture, where it took 40 days to degrade 20 mM of propionate (0.5 mM per d) (Scholten and Conrad 2000). A pure culture using *Syntrophobacter sulfatireducens*, which is the best performer of POB using sulfate reduction for electron transfer reactions, was the only organisms with a similar degradation rate. A quantum of 20 mM propionate was degraded in 12 – 15 days, hence a degradation rate of 1.3 – 1.6 mM per d can be calculated. Another indication for a close relatedness of the *Syntrophobacter*-like organism of the mesophilic propionate-oxidizing bi-culture to *Syntrophobacter sulfatireducens* comes from FISH and microscopy evaluation. On the one hand the *Syntrophobacter* related organism shares some similarities with *Syntrophobacter fumaroxidans*, as there is a hybridization with probe MPOB1 and no fluorescence using probe KOP1 (Felchner-Zwirello 2013), however, on the other hand the culture was not able to grow on fumarate. Thus that would be another argument, that *Syntrophobacter sulfatireducens*, which also gives a hybridization signal using probe MPOB1, is the closest related organism. The comparison of microscopic images of an early state culture with a culture, which already had degraded large amounts of propionate confirm the findings of Felchner-Zwirello et al. (2013), who reported the occurrence of large aggregates in the later growth phases. This goes hand in hand with the reductions of the interspecies distance, which is especially important for the syntrophic cultures.

4.2 Co-digestion of bread with biowaste

When methanogenesis was disturbed by overload, and hence the pH dropped (after day 115), the decrease of the numbers of *Archaea* was stronger than that of *Bacteria*. Members of the domain of *Bacteria* were, however, not further differentiated, so changes of different subgroups within this domain, for instance of numbers of different VFA-forming bacteria could not be seen by FISH analysis. Other authors, who have looked into taxonomic subgroups in their analysis, where hampered by the fact, that organisms like acetate producers are found in different subgroups, e.g. within acidogenic and acetogenic bacteria, so the use of 16s rRNA approach is of limited success (Hori et al. 2006, Lerm et al. 2012).

“Varying biowaste or co-substrate composition for anaerobic digestion may cause changes of VFA spectra and of ammonia concentrations in the digester, and therefore lead to changes of the microbial flora” (Li et al. 2015b). As in many reactors 60 % of the methane from biogas processes was produced by aceticlastic methanogens (Gallert and Winter 2005). Protein-rich feed material, which will bring high ammonia levels in the reactors, is a relevant factor of AD. Fatty acid and ammonia concentrations have a strong effect on methanogenic community, as *Methanosaeta* sp. populations are especially sensitive to ammonia (Supaphol et al. 2011, Mata-Alvarez et al. 2014). When acetate concentrations were high in biogas digesters *Methanosaeta* sp. may be out-competed by *Methanosarcinaceae*, which become dominant at acetate concentrations above 250 – 500 mg COD/L (De Vrieze et al. 2012). When growth of *Methanosaeta* sp. and *Methanosarcinaceae* was inhibited by even higher VFA concentrations, especially when n-butyrate was present, hydrogenotrophic methanogens and AOB must fill the gap (Supaphol et al. 2011). Mata-Alvarez et al. (2014) also reported an inhibition of aceticlastic methanogen at high levels of ammonia and/or high VFA concentrations. When hydrogenotrophic methanogens take over methanogenesis, syntrophic acetate-oxidizing bacteria (SAOB) are essential, as in the absence of *Methanosarcinales* only SAOB can oxidize acetate to H_2 and CO_2, which are converted to methane by the hydrogenotrophic methanogens. Acetate conversion to methane and CO_2 by *Methanosaeta* or *Methanosarcina* sp. is no longer possible under these conditions. The produced H_2 is syntrophically consumed by hydrogenotrophic methanogens, which is important to keep the acetate degradation of SAOB thermodynamically favorable (Müller et al. 2013). In another paper similar observations were made, when ammonia levels in semi-continuously operated experimental laboratory-scale biogas reactors were elevated step by step (Westerholm et al. 2011). At ammonia levels higher than 0.8 g NH_4^+-N per L aceticlastic acetate degradation was hampered and syntrophic acetate oxidation took over, as also demonstrated in a control reactor with low ammonium content, where no such shift of the population was observed (Westerholm et al. 2011). In one article the 16S rRNA gene concentrations of *Methanosaetaceae* was measured: At an NH_4^+-N concentration of 0.9 g per L in the reactor *Methanosaetaceae* decreased about 104-fold and hence acetate was converted by SAOB (Ren et al. 2014). The results of reactors 1 and 2 confirmed all this findings. As the concentration of ammonium was close to but under the threshold concentration of 1 g per L in our reactors for anaerobic digestion of FBS ± WBS the dominating aceticlastic methanogen was *Methanosaeta* sp., similarly as reported from microbial community analyses of anaerobic reactors operated with mixed wastes (Supaphol et al. 2011). Some unknown components in the RBS, which apparently were not present in WBS must have led to a shift within the POB to less *Pelotomaculum* sp. and significantly more *Syntrophobacter* and *Smithella* sp. The reverse effect was observed by Shigematsu et al. (2006) for dilution rates of > 0.1 per d and, when feeding was changed to FBS only after reactor crash (Figure 21).

4.3 Full scale re-start after revision

Re-start of the biogas plant Karlsruhe Durlach after a revision of the biogas reactor was accompanied by Gallert and Winter (2008). Biogas production and volatile fatty acid spectra were analyzed, but at that time no population analyses were made. Comparing a new startup procedure after a recent revision with the former start-up procedure for the same wet anaerobic digester with the same source of biowaste revealed that accumulation of acetic and propionic acid and degradation was different. In the work of Gallert and Winter (2008) it took 12 days for the degradation of accumulated acetic acid and 29 days for the degradation of accumulated propionic acid, respectively, whereas during the actual start-up acetate was degraded within 12 days and propionate within 13 days. The maximum acetic acid concentration was lower, however, in the earlier re-start (1.8 g per L, Gallert and Winter 2008) as during the recent re-start (1.3 g per L, Moertelmaier et al. 2014) after revision of the full-scale plant. In another work dealing with the start-up of a digester VFA concentrations remained very low and acetate and propionate dropped within 10 days to values near zero (McMahon et al. 2004).

Comparing the developments of cell numbers of the *Bacteria* and *Archaea* with the concentrations of VFA leads to the conclusion that the increase of *Bacteria* during accumulation of VFA within the first 7 days (Figure 25) was caused by rapid growth of acetogenic and propionic acid forming bacteria (Moertelmaier et al. 2014). A strong increase of *Bacteria* was also seen in a batch experiment due to fast growth of acidogenic bacteria within the first 2 days (Lee et al. 2008). Other authors reported instead an increase of *Archaea* only, whereas numbers of *Bacteria* decreased (e.g. Shin et al. 2010, Griffin et al. 1998).

DAPI is a dye that reacts with all cells that contain double stranded DNA, no matter whether they are alive or dead, and thus allows to determine total cell numbers in complex samples. To be able to distinguish *Bacteria* and *Archaea* fluorescence labeled gene probes covering bacterial domains must be used. Those gene probes apparently do not detect all members of a population or a high proportion is already inactive, since in our investigation and in many examples from literature, the sum of *Bacteria* and *Archaea* cell numbers, determined by FISH did not reach the much higher level of cell numbers stainable by DAPI. As no material was removed from the full-scale biowaste reactor within the first 19 days the large difference in cell numbers counted after DAPI-staining and after FISH of cells followed by microscopy must be due to either a high number of cells, which are not covered by FISH probes or to inactive, non-stainable bacteria. Presumably both reasons contribute to the missing part of the population after FISH analysis.

Propionate oxidation by POB necessarily leads to hydrogen production, which must be consumed by sulfate-reducing bacteria or hydrogenotrophic methanogens (e.g. *Methanomicrobiales*). In any case a hydrogen partial pressure above 10^{-4} atm must be avoided (Felchner-Zwirello et al. 2013, Li et al. 2012) since propionate oxidation would become thermodynamically unfavorable at higher hydrogen partial pressure. A comparison

of propionic acid concentrations and of abundances of POB revealed a negative relationship, which means that high numbers of POB resulted in low propionic acid concentration. This was in accordance with data of McMahon et al. (2004) and similar to the developments in the reactors of Moertelmaier et al. (2014). Due to the late recognition of *Pelotomaculum* species as POB, in earlier investigations many authors did not check for them, hence POB communities were described as dominated by *Smithella propionica* (Ariesyady et al. 2007a) or *Syntrophobacter* sp. (McMahon et al. 2004). Also the dilution rate seems to play a role for prevalence of certain POB, as in a chemostat fed with propionate the dominance of *Pelotomaculum* sp. was only observed at high dilution rates of 0.3 per d (Shigematsu et al. 2006). Worm et al. (2009) found that *Pelotomaculum* and *Smithella* numbers increased and *Syntrophobacter* sp. decrease after a long incubation period in a tungsten, molybdenum and selenium limited UASB reactor. Another reason could surely be found considering the composition of POB of the inoculum and of the substrate, which showed a majority of *Pelotomaculum* sp. already. Thus it would be difficult for *Smithella* and *Syntrophobacter* sp. to outcompete *Pelotomaculum* sp. at conditions which apparently favored *Pelotomaculum* sp. (Figure 28, Moertelmaier et al. 2014). *Syntrophobacter* sp. was the only POB group which increased during the phase of propionate accumulation in the biowaste. As *Syntrophobacter* sp. represents only a small minority of POB, the increase was too weak to compensate the losses of the other taxa and the number of total POB fell. Hence the degradation capacity of the microbial community decreased.

High numbers of *Methanosaeta* sp. during start-up went along with findings by other authors, who reported a stable pH and a quick degradation of VFA (Karakashev et al. 2005, McMahon et al. 2004, Supaphol et al. 2011) In many different recent publications *Methanomicrobiales* were reported to contribute a major part to the methanogens in various anaerobic systems (Angenent et al. 2002, Shin et al. 2010), or in acidogenic digesters with a low pH (Liu et al. 2002). Acetate oxidizing bacteria (AOB) can form a syntrophic association with hydrogenotrophic methanogens , hence a competition of AOB and acetoclastic methanogens for the available acetate in anaerobic digesters was proposed (Schnürer et al. 1999). A closer look at the cell numbers in the used substrate – fresh, undigested biowaste- revealed that 50 % to the methanogenic population belonged to the *Methanomicrobiales* which formed also the major methanogenic group after digestion (Moertelmaier et al. 2014). The order of the *Methanobacteriales* did not play any role during start-up, which may have been due to the neutral pH and relatively low VFA concentrations. On the other hand *Methanobacteriales* would be able to outcompete other methanogens at low pH and high VFA concentrations (Blume et al. 2010).

4.4 Propionic acid co-digestion with biowaste

In reactor 1, which was fed with biowaste alone, the numbers of *Bacteria* and of *Archaea,* obtained between day 16 and day 25, did not represent the status of a biowaste-alone fed reactor, as this numbers showed the influence of the start-up, e.g. VFA were still present at that time (Figure 31). Hence the numbers obtained between day 31 and 45 were used for comparison with the other reactors. In reactor 2 growth of *Bacteria* was much faster than

that of *Archaea*. “When the OLR of reactor 2 was increased from 12 kg COD m^{-3} d^{-1} to 13 kg COD m^{-3} d^{-1} by propionate addition, the community density of *Bacteria* reached its maximum within a few days and then remained stable, even when the OLR was further increased with propionate addition” (Li et al. 2015a). Growth of *Archaea* was slower, however the number of *Archaea* increased by feeding higher amounts of propionate (Figure 35a). In the first days no clear difference could be seen if the numbers of *Archaea* in reactor 1 and 2 were compared, but at a feeding of 16.5 kg COD m^{-3} d^{-1} numbers of *Archaea* began to be higher in reactor 2 than in reactor 1. Hence also the proportions of the two domains changed with time from 18 % at an OLR of 13 kg COD m^{-3} d^{-1} (including 1kg $COD_{propionate}$ m^{-3} d^{-1}, day 32) to 40 % at an OLR of 18 kg COD m^{-3} d^{-1} (including 4 kg $COD_{propionate}$ m^{-3} $d^{-}1$, days 100 – 120), whereas in reactor 1 the average percentage of *Archaea* between days 31 – 45 was 25 %. In reactor 3 the proportion of *Archaea* was also very high, comparable to the proportions of *Archaea* in reactor 2 from day 56 on for a feeding > 14 + 2.5 kg COD m^{-3} d^{-1}. During the last days of the experiment the proportion of *Archaea* reached its maximum with 51 %. Comparison with the work of other authors is difficult, as no manuscript could be found, which used biowaste and propionate as substrate at the same time. However In digesters with a similar substrate but without addition of propionate (e.g. in the report of McMahon et al. (2004), where a simulated synthetic organic fraction of municipal solid waste was used as substrate), 21 – 23 % of total prokaryotes were *Archaea*. Wang *et al.* (2006) found a proportion of *Archaea* between 12 – 18 % of the total number of prokaryotes in a digester fed with pre-treated food waste. Angenent et al. (2002) also reported a low proportion of only 15 % *Archaea* within the total prokaryotes, when swine manure was digested. Other researchers ran reactors with propionate as the sole source of carbon and found a proportion of *Archaea* of 64 % within the total prokaryotes in a continuously operated, solely propionate fed chemostat (Shigematsu et al. 2006). A plausible explanation would be, that with the addition of propionate, carbon enters the anaerobic food web at a close to the end point of degradation, if compared with more complex substrates. Biowaste is a complex substrate, containing sugars and proteins among others, hence more different species are needed to completely degrade the diverse substrates. On the other hand “in digesters with propionate feeding, only POB and eventually acetate-oxidizing bacteria (AOB) and H_2/CO_2, as well as acetate-utilizing methanogens, are required” (Li et al. 2015a). “Therefore, it seems plausible that the proportion of *Archaea* increased with an increasing OLR by propionic acid addition into the reactor“ (Li et al. 2015a). A further confirmation would be the calculation of biogas per kg COD m^{-3} d^{-1} added. Per kg $COD_{propionate}$ 5 – 6 L biogas m^{-3} d^{-1} were produced, whereas 2.4 – 2.8 L biogas were produced from 1 kg $COD_{biowaste}$ m^{-3} d^{-1} (Table 15).

Table 15: Calculation of biogas produced per 1 kg COD m^{-3} d^{-1} in the reactors. a: days 0 – 15 start-up phase; b: days 16 – 50 steady state; c: first week biowaste alone was fed. Adapted from Li et al. (2015a)

OLR biowaste	OLR propionate	Biogas L d^{-1} (kg COD m^{-3} d^{-1})$^{-1}$
	Reactor 1	
12[a]	0	2.4
12[b]	0	2.7
	Reactor2	
12	0	2.5
12	1	2.5
12	1.5	2.9
14	2.5	2.5
14	3	2.8
14	4	2.7
	Reactor 3	
11[c]	0	1.8
11	3	2.8
3	3	6.2
11	0	2.5
5	5	5.2

In reactor 2 “a strong positive correlation (Pearson correlation 0.86) was found between numbers of *Methanomicrobiales* and biogas production” (Li et al. 2015a, Table 16). Hence the more propionate was added, the more biogas was generated and the higher numbers of *Methanomicrobiales* could be detected. Numbers of Methanosarcinales, however, did not correlate with the amount of added propionate. This would indicate that the methane originating from propionate degradation was produced by the hydrogenotrophic *Methanomicrobiales* community. As there was no acetate accumulation detectable in the reactor effluent “and numbers of acetate-fermenting *Methanosarcinales* remained almost constant at increasing OLRs maintained by propionate addition , AOB may have been active, delivering CO_2 and H_2 for the *Methanomicrobiales* community” (Li et al. 2015a). When the number of *Methanosarcinales* stagnated, then a plausible theory would be that acetate was consumed by AOB. Hori et al. (2006) reported a collaboration between AOB and hydrogenotrophic methanogens for acetate cleavage to CO_2 and hydrogen. In another work a positive correlation between numbers of *Methanoculleus* sp., a genus of *Methanomicrobiale*s, and the number of *Pelotomaculum* sp. (Pearson correlation 0.98) was documented (Li et al. 2015a, Table 16). As in this work it was only searched for methanogenic orders, the number of *Methanoculleus* sp. per se was unknown, but a positive correlation, between *Pelotomaculum* sp. and the number of *Methanomicrobiales* (Pearson correlation 0.79) was seen. This positive correlation could, however, not be seen in every case. During start-up of the full scale digester in Karlsruhe Durlach for instance high numbers of *Pelotomaculum* sp.

and low numbers of *Methanomicrobiales* were found (Moertelmaier et al. 2014). "A syntrophic association of *Pelotomaculum* sp., AOB and members of *Methanomicrobiales* is another possibility but not a "conditio sine qua non" for growth on propionate" (Li et al. 2015a, Moertelmaier et al. 2014). In reactor 3 there was a different situation as all methanogenic taxa grew in equally high numbers, although variations occurred within a week. Another difference was that the first sample contained a majority of *Methanosarcinales*. That could be the reason, why reactor 3 did not show a similar development like reactor 2, where *Methanomicrobiales* mainly contributed to the growth of methanogens.

Compared with the POB numbers in reactor 1, the numbers of POB in digesters that were fed with biowaste + propionate were slightly higher (reactors 2 and 3): In reactor 1 (1.3 – 3) × 10^8 POB per mL were found from day 24-45, whereas in reactor (2.5 – 6) × 10^8 POB per mL were detected (contribution of propionate addition to the total COD: 1 – 2.5 kg COD m^{-3} d^{-1}) (Figure 34b, Figure 35b). After a necessary repair in reactor 2 (4.8 – 9.4) × 10^8 POB per mL were found at the highest propionate contribution to the OLR (3 – 4 kg COD m^{-3} d^{-1}), although not all propionate was degraded at that time (Figure 32, Figure 35b). In reactor 3 no propionate could be detected from day 17 on. At this time (day 20 and 22) numbers of POB were 9.1 and 8.9 × 10^8 POB per mL, respectively (Figure 36b) Thus numbers were similar high as numbers in reactor 2 at a propionate addition of 3 – 4 kg COD m^{-3} d^{-1}. When feeding was changed in reactor 3 numbers of POB were lower (4.8 – 6) × 10^8 POB per mL, similar to numbers in reactor 2 at a propionate addition of 2.5 kg COD m^{-3} d^{-1} (Figure 35c, Figure 36c). In the paper of Li et al. (2014b) degradation experiments were conducted, taking samples of reactor effluent at different times and incubate them separately in serum bottles with freshly added propionate, to determine the propionate degradation potential at that time. Cell numbers of POB (and *Pelotomaculum*) corresponded well with the findings of this experiment.

Table 16: Pearson's correlation of cell numbers, biogas production and degradation rates in reactor 2. Adapted from Li et al. (2015a)

	Archaea	*Methano-microbiales*	*Methano-sarcinales*	*Methano-saeta*	*Peloto-maculum*	Degradation rates
Biogas L d^{-1}	0.80	0.86	0.72	0.55	0.80	0.99
Pelotomaculum	0.63	0.79	0.49	0.29	1.00	0.84
POB	0.68	0.83	0.53	0.31	0.98	0.85

Like in all other experiments carried out with the biowaste feed from the full scale digestor plant of Karlsruhe Durlach the most numerous POB were *Pelotomaculum* sp. followed by significantly less *Syntrophobacter* sp. and *Smithella* sp. (Figure 35c). The reasons for that were partly discussed in chapter 4.3, as e.g. influence of inoculum (Moertelmaier et al. 2014). Another important point would be, that the diversity of POB was higher in reactor 2 than in reactor 3, although the maximum numbers of total POB were similar. This was mainly due to the higher numbers of *Syntrophobacter* sp. at a propionate addition of 3 kg COD m^{-3}

d^{-1}. At this OLR *Syntrophobacter* sp. represented 1.4 – 2.7 % of *Bacteria*, increasing to 5.2 – 6 % at an OLR of 4 kg COD m^{-3} d^{-1} (Figure 37a). When the OLR was increased high concentrations of *Pelotomaculum* sp. left the reactor with the effluent. Possibly *Pelotomaculum* sp. were hampered in growth, and *Syntrophobacter* sp. filled the gap, without being able to use the whole potential of propionate (Li et al. 2015a). As it was mentioned in chapter 1.3 the mesophilic species of *Pelotomaculum* are considered specialists among POB and may not have had the possibility of fermentative growth on e.g. pyruvate or fumarate. When propionate oxidation becomes unfavourable, because methanogens cannot consume hydrogen fast enough, organisms like *Syntrophobacter fumaroxidans* have an advantage, due to their possibility of fermentative growth (Table 3). In reactor 3, where the maximum cell contribution of *Syntrophobacter* sp. was 2 % and *Pelotomaculum* sp. made up more than 20 % of the *Bacteria*, no propionate was left in the effluent. This would confirm the explanation above (Figure 37b). *Smithella* sp. was even less able to compete with *Pelotomaculum* sp. When *Pelotomaculum* sp. and *Syntrophobacter* sp. began to grow already at an OLR of 13 kg COD m^{-3} d^{-1} (12 kg $COD_{biowaste}$ m^{-3} d^{-1} + 1 kg $COD_{propionate}$ m^{-3} d^{-1}), *Smithella* sp. began to grow only when an OLR of 1.5 kg $COD_{propionate}$ m^{-3} d^{-1} was supplied and "then approached numbers of *Syntrophobacter* sp." (Li et al. 2015a). However, at higher OLR numbers of *Smithella* sp. decreased again to much lower final cell numbers (< 10^7 mL^{-1}) than *Pelotomaculum* sp. or *Syntrophobacter* sp. (> 10^8 mL^{-1}; Figure 6c, days 90 – 120). Thus *Smithella* sp. seem not to be able to compete with the dominant *Pelotomaculum* sp. at propionate levels lower than 1.5 or higher than 2.5 kg $COD_{propionate}$ m^{-3} d^{-1} (Li et al. 2015a).

4.5 Mesophilic and thermophilic dry fermentation

For comparison of our data with literature reports the work of Zahedi et al. (2013) is suitable, as the same procedures for the determination of cell numbers were used. The digesters of Zahedi et al. (2013), however, were operated with a semi-continuous feeding systems. Thus the cell numbers of *Bacteria* and of *Archaea* were much higher than in our "box type fermenter", from which the investigated samples were collected (Li et al. 2014a). Numbers for *Bacteria* and *Archaea* fluctuated between (5.1 – 9.9) x 10^9 and (1 – 2) x 10^9 cells per ml in the semi-continuous feed approach of Zahedi et al 2013. The numbers for the two bacterial domains in the work of Zahedi et al. (2013) were 3 – 6 fold and 2 – 3 fold higher than in the actual work and the related paper (Li et al. 2014a). Reasons for that may be caused by the beneficial effect of stirring and mixing in the reactor operated by Zahedi et al. (2013). "Since our DAD reactors were not permanently stirred reduced mass transfer may have been the reason for reduced cell growth. This is the "reality" in all dry anaerobic digesters without process water recycling"(Li et al. 2014a).

As a vast majority of *Methanosarcinales* was determined in DAD samples, it has to be considered, that this order comprises many different genera, including not only *Methanosarcina* sp. but also *Methanococcoides*, *Methanolobus* and *Methanohalophilus* sp. (Raskin et al. 1994). On the other hand there are many examples in literature for

methanogenic communities with poor diversity. Chu et al. (2010), who determined the methanogenic community of mesophilic and thermophilic AD of food waste, for example reported a strong dominance of *Methanosaeta concilii* (72 %) in a mesophilic community and an even higher dominance of *Methanosaeta thermophila* (98 %) at thermophilic conditions. In another work similar observations were made: the diversity of methanogens decreased and 96.4 – 99.1 % dominance of *Methanosarcina* sp. was seen instead (Cho et al. 2013).

From the actual results it has to be assumed, that *Methanosaeta* sp. did not play any role in the different DAD reactors. A plausible reason for this could be found in a past paper, in which high acetate concentrations favored *Methanosarcina* sp. (Hori et al. 2006). Indeed the "high acetate concentrations" were much higher than 1 g per L as in our digesters; hence they were beyond the optimum for *Methanosaeta* sp. (Karakashev et al 2005). In all works with a dominance of *Methanosaeta* sp. (e.g. Chu et al. 2010, Montero et al. 2009) acetate concentrations were much lower than in the actual work (Li et al. 2014). Another remarkable fact is that there was methanogenesis even at acetate concentrations above 8 g per L, which occurred after 21 d. This concentration should prevent activity of both *Methanosarcina* and *Methanosaeta* sp. (McMahon et al. 2004). It was however hypothesized earlier, that due to the heterogeneous environment and the difficulty as well as the lack of mixing, in niches, where pH is higher and VFA concentrations are lower *Methanosarcina* may survive and may be metabolically active (Abbassi-Guendouz et al. 2013).

The hydrogenotrophic taxa *Methanomicrobiales* and *Methanobacteriales* played a minor role during DAD. One reason could be, that we did not sample during the first days of incubation. It was reported that hydrogenotrophic methanogens usually were dominant after start of DAD at high VFA levels (Montero et al. 2009). There were cases of high numbers of *Methanobacteriales,* when extremely high VFA concentrations and a pH below 6 were prevalent (Blume et al. 2010). In other works with less extreme VFA levels and a pH between 6 and 7 *Methanomicrobiales* outnumbered the *Methanobacteriales* (Garrity and Holt 2001). As a pH drop below 6 did not occur in the DAD fermenters *Methanobacteriales* did not find an appropriate environment for growth, in which they could successfully compete with the other more numerous methanogenic orders. This is in agreement with our results, as pH never decreased below a value of 6.5 and thus only a minimal number of *Methanobacteriales* could be detected.

Apart of the actual work and the published papers linked to it (e.g. Li et al. 2014a) very little is known about POP numbers in DAD. There is one other work, in which POB in DAD reactors were counted (Zahedi et al. 2013). In the study of Zahedi et al. (2013), however, only the probe synbac824 was used, although the samples were collected from a thermophilic reactor. As mentioned earlier all known members of the genus *Syntrophobacter* cannot grow under thermophilic conditions. But the numbers of *Syntrophobacter* sp. reported by Zahedi et al. (2013) were much higher: Between 6 and 15 % of *Bacteria* belonged to *Syntrophobacter* sp. and the concentration of propionate was lower than in all DAD reactors of Li et al. (2014a), namely 0.06 – 1.3 g per L (Zahedi et al. 2013) versus 0.2 – 21 g per L (Li et al. 2014a) In the actual work there were also *Syntrophobacter* sp. found in the thermophilic

reactors, especially in the assays with high dry mass, in which much less biogas was formed. It can be hypothesized that these synbac824 positive cells were fragments, as the inoculum, which was used for the thermophilic experiments, originated from a mesophilic full scale digester and hence was exposed to much lower temperatures earlier. As the material used for the thermophilic assays was kept as a batch incubation for at least 300 d at 55°C, fragments of *Syntrophobacter* sp. from the inoculum may have been able to persist under the not favorable temperature for them for a long time. There is a negative linear relationship between propionate concentrations and the absolute numbers of POB in the mesophilic assays, indicating that the higher the number of POBs the lower the propionate concentration was
Figure 51). If the numbers of thermophilic POB are added then no clear linearity could be seen, especially because the numbers of POB for the reactor containing 25 and 30 % DM are too low. Maybe there are still yet unknown thermophilic POB. Thus POB are essential to prevent acidification by high VFA contents.

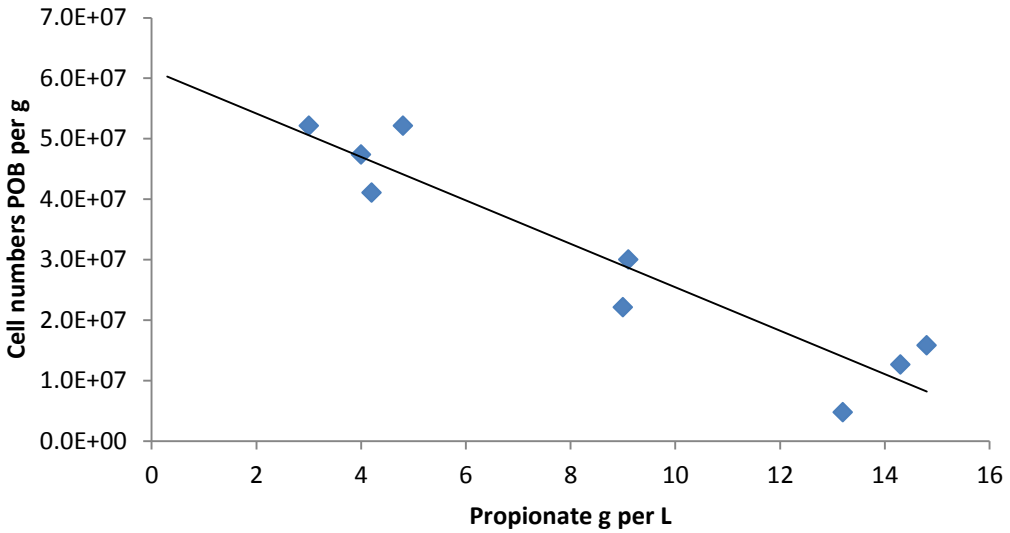

Figure 51: Linear relationship between cell numbers of POB per g and propionate concentration (g per L).

4.6 Probe evaluation

Since oligonucleotide probes based on 16S rRNA sequences were applied widespread for identifying bacteria in mixed cultures, there is a lot of literature on introducing new probes. However there is not always a detailed description of the results of formamide serial treatment and measurements of corresponding fluorescence intensities (e.g. Narihiro et al. 2012, Ariesyadi et al. 2007b). In the work of Daims et al. (1999) the same software was used for analysis as by us, thus the results should be comparable. The "hardware" of Daims et al. (1999) was, however, far better (confocal laser microscopy) hence comparison is difficult. It

could be seen that the fluorescence in general is twice as high as even in our positive control. Probe Eub388, which was used as positive control in the actual work is known to work at a very broad range of possible formamide concentrations and is therefore the ideal probe for positive controls in FISH experiments (Daims, et al.1999). There is a more recent work, which delivers detailed information on the behaviour of different designed probes (Lücker et al.2007). It can be seen that even a one base mismatch can lead to a complete different behaviour of the probe resulting in a strong decline of fluorescence. On the other hand there is also some variation in the maximum fluorescence intensity, which can be produced by different organisms with perfect matching sequence (Lücker et al. 2007).

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