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EVALUATION OF THE EFFECTIVENESS OF BIOGAS PRODUCTION WITH A COMBINATION OF PHYSIOLOGICAL AND MOLECULAR APPROACH

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DISSERTATION

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Abstract of dissertation

EVALUATION OF THE EFFECTIVENESS OF BIOGAS PRODUCTION WITH A COMBINATION OF PHYSIOLOGICAL AND MOLECULAR APPROACH

by Domen Novak

In order to test new and less used difficultly degradable substrates for biogas production, the BMP assay was optimized for developing a stable anaerobic methanogenic process as well as for routine monitoring of the process stability. BMP assay combines molecular approaches (PCR, T-RFLP, microarrays, cloning and sequencing) and already known methods for testing methane production and was used for bio-monitoring of the degradation process effectiveness, community dynamics and structure-function relationship of methanogenic and also bacterial microbial community in the event of substrate change.

With BMP assay we estimated the effects of a novel and difficult degradable substrate (brewery spent grain) microbial community in three distinct biomasses. As a result, a general positive relationship between the flexibility of archaeal community and its initial biogas production rate was identified as a novel predictor of inoculum suitability in digester start up.

Further, we determined the possibility of cyanide degradation in two distinct and contrasting biomass (continuous stirred-tank reactor - CSTR) and (upflow anaerobic sludge blanket - UASB) samples. Experiments with CSTR biomass revealed that metal cyanide complexation was probably the main reason that almost none of cyanide was removed during the anaerobic incubation. Research showed that anaerobic microbial consortia could be adapted to reduced metal-complexed cyanide toxicity, but could still not use cyanide as carbon or nitrogen source for growth.

UASB microbial biomass was successful in degradation of cyanide without noticeable inhibition at cyanide ion concentrations up to up to 5 mg/L. At a higher initial concentration of cyanide (8.5 mg/L), a lag phase, which lasted 21 days, was detected. Following the lag phase, normal methanogenesis at rates comparable to those in the positive controls were observed. Based on the results of the study, we concluded that anaerobic degradation of cyanide observed in our experiment was most probably a combination of simultaneous abiotic and biotic processes.

Phylogenetic analyses of bacterial and archaeal microbial communities that are involved in the cyanide degradation under anaerobic conditions indicated that bacterial phylum *Firmicutes* was the crucial microbial group in cyanide degradation, since their frequency significantly increased in cyanide amended samples. Archaeal group *Methanosarcina* could be associated with the effects caused by the cyanide degradation.

Keywords: BMP assay, biogas, anaerobic digestion, microbial community, cyanide, brewery spent grain, *Firmucutes*, *Methanosarcin*a

Povzetek disertacije

VREDNOTENJE UČINKOVITOST BIOPLINSKE PROIZVODNJE S KOMBINACIJO FIZIOLOŠKEGA IN MOLEKULARNEGA PRISTOPA

Domen Novak

Z namenom, da bi lahko testirali težko razgradljive in zaradi teh lastnosti manj uporabljane substrate v bioplinski proizvodnji, smo optimizirali test biometanskega potenciala (BMP) za načrtovanje stabilnega anaerobnega metanogenega procesa kot tudi za rutinsko spremljanje njegove stabilnosti. Vzpostavljeni sistem je kombinacija molekularnega pristopa (PCR, T-RFLP, mikromreže, kloniranje in sekvenciranje) in običajnih, že vpeljanih metod za preizkušanje metanske produkcije. Vzpostavljeni sistem je bil nadalje uporabljen za bio-monitoring učinkovitosti procesa razgradnje, dinamike mikrobne združbe in razmerij med strukturo in funkcijo metanogenih arhej in bakterij v primeru zamenjave substrata.

Z vzpostavljenim sistemom smo ocenili učinke novih, slabše razgradljivih substratov (pivske tropine) mikrobne združbe v treh različnih mikrobnih biomasah. Rezultat je bil splošno pozitiven odnos med prilagodljivostjo arhejske mikrobne združbe in njeno začetno stopnjo proizvodnje bioplina, in kot tak prepoznan kot nov pokazatelj primernosti inokuluma pri zagonu anaerobnega reaktorja.

Nadalje smo določili možnost anaerobne razgradnje cianida z dvema različnima biomasama (CSTR in UASB). Poskusi z biomaso CSTR so pokazali, da je bilo formiranje kovinsko cianidnih kompleksov verjetno glavni razlog za majhno količino odstranjenega cianida v času anaerobne inkubacije. Raziskave so pokazale, da se anaerobna mikrobna združba lahko prilagodi toksičnosti kovinsko cianidnih kompleksov pri tem pa ne more uporabljati cianida kot vir ogljika in dušika za svojo rast.

Pri biomasi UASB je bila razgradnja cianida uspešna in je potekala brez opazne inhibicije do koncentracije 5 mg/L cianida. Pri višji začetni koncentraciji cianida (8.5 mg/L) je bila opažena faza zamika (lag), ki je tajala 21 dni. Po končani fazi zamika je metanogeneza potekala normalno, brez inhibicije, primerljivo s pozitivno kontrolo. Glede na rezultate raziskave smo zaključili, da je anaerobna razgradnja cianida v našem primeru verjetno kombinacija hkratnega biogenega in abiogenega procesa, vendar ne izključno biološka.

Filogenetske analize bakterijskih in arhejskih mikrobnih združb, ki so vpletene v razgradnjo cianida pod anaerobnimi pogoji, so nakazale, da je bakterijsko deblo *Firmicutes* ključna mikrobna skupina pri razgradnji cianida, saj se je njihova zastopanost signifikantno povečala v vzorcih z dodanim cianidom. Arhejska skupina *Methanosarcina* bi lahko bila povezana z učinki, ki so povzročeni s predhodno razgradnjo cianida.

Ključne besede: test (BMP), bioplin, anaerobna razgradnja, mikrobna združba, cianid, pivske tropine, *Firmucutes, Methanosarcina*

ABBREVATIONS

ATA	Anaerobic toxicity assay
BMP	Biochemical methane potential
BWW	Brewery wastewater
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DGGE	Denaturation gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FBY	Fresh brewery yeast
FISH	Fluorescence in situ hybridization
HBY	Hydrolyzed brewery yeast
MCC	Metal cyanide complex
NMDS	Non-metric multidimensional scaling
NanoSIMS	Nano secondary ion mass spectrometry
PCR	Polymerase chain reaction
Q-PCR	Quantitative polymerase chain reaction
SIP	Stable isotope probing
SSCP	Single-strand conformation polymorphism
TCN	Total cyanide
TOC	Total organic carbon
TRFLP	Terminal restriction fragment length polymorphisem
TS	Total solids
UPGMA	Unweighted pair-group method with arithmetic averages
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acids
VS	Volatile solids

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1 INTRODUCTION

Organic waste typically originates from plant or animal sources. It represents the biggest fraction of municipal waste in Europe. It is estimated that 30 to 50 % of all waste in EU is organic. It is broken down by microorganisms. The main environmental threat from organic waste is the production of methane and carbon dioxide from landfills, which accounted for some 3% of total greenhouse gas emissions in the EU-15 in 1995 (European Commission Environment, 2011). Three decades ago biological treatment of waste was limited to a few composting plants for treating unsorted organic waste (Alvarez, 1999). Meanwhile anaerobic treatment of organic waste became topical as an alternative method due to the oil crisis in the seventies of the past century. Germany was the pioneer in this field. In the 1990s biogas technology was even more stimulated by the profitability of using power derived from biogas and because of the EU legislation (Waste Avoidance and Management act), which resulted in higher costs for disposal of organic waste. In the year 2000 the law for renewable energies became effective and enabled subsidization of the power derived from biogas plants. In the last decade biogas plant production has been continuously rising with Germany as the leading country in biogas production having established approximately 5.000 biogas installations.

Anaerobic methanogenic fermentation is a complex microbial process that takes place in sequential stages of decomposition and is carried out by different groups of microorganisms, acting in syntrophic interaction (the exchange of substrates and products of fermentation). Final end product of anaerobic digestion is biogas, which is mainly composed of carbon dioxide and methane and can be used as an energy source.

Anaerobic treatment of organic waste is energetically and financially more economical in comparison to the aerobic treatment and produces less waste biological sludge (Montero et al., 2007). Till today, many research groups have developed different procedures for anaerobic treatment with the use of various organic substrates (Foster-Carneiro et al., 2007, 2008).

For optimization of methane production in anaerobic digestion empirical methods are most commonly used through measurements of short chain fatty acids, temperature, pH, hydrogen partial pressure, redox potential, concentration of ammonia, biomass load, specific surface of material and other parameters (Ahring et al., 1995, Leiu et al., 2009).

Modern molecular biology techniques in microbial ecology provide new insight into the structure of microbial communities and attempt to link it to their function. Phylogenetic analyses of anaerobic sludge microbial community based on the 16S rRNA genes identified several methanogenic archaea, syntrophic bacteria, sulfate reducing bacteria and a number of unknown populations to coexist in anaerobic reactors (Plugge et al., 2002, Imachi et al., 2002, de Bok et al., 2005, Kobayashi et al., 2008).

However, a few studies of microbial communities in anaerobic processes focused on the composition including also the changes in the structure of microbial communities through various stages of anaerobic process at the level of 16S rRNA gene or different functional genes (Narihro, et al., 2007, Hori et al., 2006). It has been shown that differences between microbial communities grown with different substrates can be observed on the level of 16s rRNA gene. This indicates that information from molecular analyses could contribute considerably to a better understanding of changes during the anaerobic digestion process and identify possible causes of the process failure that may occur in response to differences in substrate nature, amount, loading rate, particle size or state. Implementation of molecular techniques to the standard optimization procedures would thus enable faster and more accurate set up and maintenance of biogas production.

It has been long known that the most successful biogas reactors operate at the constant flow of one substrate type and that any change in the substrate type leads to instability of the process (Chen et al., 2008). Due to increasingly strict environmental policy, waste disposal methods have been constantly changing. As of 2011, the requirement has been established to separate biodegradable waste from other waste and to treat the biodegradable waste properly (e.g. composting, anaerobic decomposition) thus challenging the companies managing biogas bioreactors with

highly divergent nature, concentration and physical state of substrate mixtures (e.g. waste from the production of food, organic municipal waste, green trim, ...etc). Such substrates have not been used before in biogas production and represent a new or unknown substrate to the residing microbial communities. Disturbances of anaerobic digestion process, which are due to differences in the substrates or substrate concentration, can in the worst-case lead to a shutdown of the gas production. The consequent adaptation to the new conditions and subsequent re-start of biogas production can last up to three or even more weeks resulting in higher (unsustainable) operating costs (Deublein et al., 2008). To ensure undisturbed and efficient production of methane and thus the removal of waste, it is necessarily to test any such unproven or unknown substrate by a rapid, reliable and economically affordable lab scale test before being used in the anaerobic degradation process on the full-scale level. Such a test can verify the biodegradability of a substrate with a precisely defined microbial biomass. On the basis of the test results one can evaluate biogas production and thus define appropriate parameters to reduce the risk of process failure. Numerous industries generate not only less degradable but also toxic substrates that can be used for biogas production. In that case long and stepwise adaptation of microbial community is needed and numerous precautions integrated into original standard procedures.

One of commonly found pollutants in organic waste is cyanide, a carbon-nitrogen radical (CN^-). It is commonly found as a contaminant in wastewaters from metal finishing industries, mining, pharmaceuticals and food industry. The industrial effluents generally contain between 0.01 and 10 mg/L of total cyanide, meanwhile in unpolluted rivers, lake and other surface waters cyanide concentrations are 100 – 500 times lower and range from 0.0001-0.05 mg/L (Wild et al., 1994). Although cyanide is present in low concentrations in various life forms, including photosynthetic bacteria, algae, fungi, plants and foods (Dash et al., 2009), the wastewater concentrations of cyanide often exceed the amount that living creature are able to survive.

1.1 Microbial processes in anaerobic methanogenic fermentation

Anaerobic fermentation is a natural biological process for the decomposition of organic material in the absence of air. The process takes place in four strongly linked phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig. 1; Demirel and Schrerer 2008). Each phase is carried out by different groups of microorganisms acting syntropically.

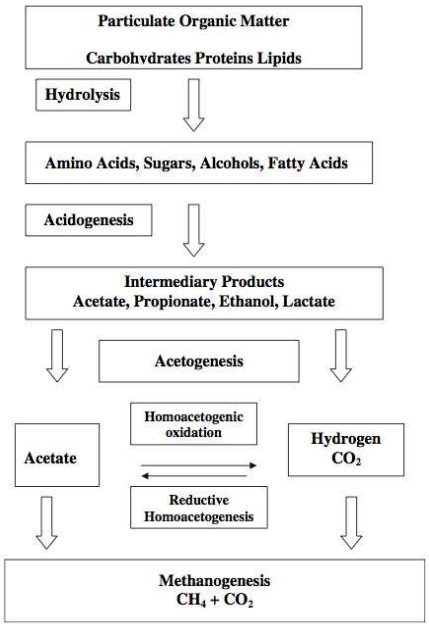

Fig. 1: *Anaerobic conversion of biomass to methane (Demirel and Scherer, 2008)*

Partially decomposed organic matter is first hydrolyzed by hydrolytic bacteria. In this phase undissolved compounds (proteins, fats, cellulose) are degraded into monomers by exoenzymes of facultative and obligatory anaerobic bacteria. The facultative anaerobic microorganisms consume oxygen dissolved in water and thus lower redox potential, which is necessary for the growth of obligatory anaerobic microorganisms. Degradation of carbohydrates takes place within few hours, while protein and fats are hydrolyzed within few days.

In the acidogenic phase, different facultative and obligatory anaerobic bacteria degrade the monomers formed in hydrolytic phase. During acidogenesis short chain organic acids (e.g. acetic acid, butyric acid, propionic acid, valeric acid), alcohols, carbon dioxide and hydrogen are formed. The limiting factor for acidogenesis is

hydrogen partial pressure (p_{H2}); the higher it is the fewer reduced compounds are formed. There are three pathways of degradation during the acidogenesis for different substrates. Amino acids are degraded by Stickland reaction in which organisms similar and related to *Clostridium botulinum* couple oxidation and reduction of two amino acids into organic acids. Carbohydrates are transformed to propionic acid via the succinic acid and acrylic acid pathway with formation of acetic and butyric acid. Fatty acids are degraded by β-oxidation by organisms similar and related to *Acetobacter* sp. Many of the organisms performing these steps are still unknown and uncultivated.

In the acetogenic phase, microorganisms reduce CO_2 with hydrogen to form acetic acid.

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_20 \quad (1)$$

Acetogenic bacteria are obligatory H_2 producers and acetate formation is thus only possible when hydrogen partial pressure (p_{H2}) is sufficiently low. This is enabled with constant consumption of H_2 by methanogens. Acetate and methane producers therefore live in symbiosis (syntrophism). Methanogenic archaea on the other hand can survive only at higher p_{H2} and they constantly remove the products of metabolism of the acetogenic bacteria and so keep the p_{H2} at a low level that is suitable for acetogenic bacteria. At low p_{H2} acetogens can grow and H_2, acetate and CO_2 are formed. Higher p_{H2} causes circumvention of acetogenesis and predominately volatile fatty acids (VFA) and ethanol are formed. These intermediates are further metabolized by methanogenic archaea to acetate, H_2 and CO_2. Approximately up to 30 % of the entire CH_4 originate from the reduction of CO_2 by H_2 and only 5-6 % of methane can be attributed to dissolved hydrogen.

In the fourth, last stage, methane formation takes place under strictly anaerobic conditions. Different methanogenic species degrade different substrates. There are three known methanogenic pathways: CO_2-reduction, methyl-group reduction, and the acetoclastic reaction (Garcia et al., 2000).

In the case of **hydrogenotrophic methanogenesis** methanogenic archaea use hydrogen as electron donor to reduce CO_2 to methane by the following reaction:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad (\Delta G°'=-136 \text{ kJ mol}^{-1}CH_4) \qquad (2)$$

Some rare species of hydrogenotrophic methanogens are capable also of formate reduction (reduction of C in formate to CH_4):

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O \qquad (\Delta G°'=-130 \text{ kJ mol}^{-1}CH_4). \qquad (3)$$

Methylotrophs use methyl groups of methanol, methylamines or dimethylsulphides. External electron donor is usually hydrogen:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \qquad (\Delta G°'=-104 \text{ kJ mol}^{-1}CH_4), \qquad (4)$$

$$4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+ \qquad (\Delta G°'=-75 \text{ kJ mol}^{-1}CH_4), \qquad (5)$$

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \qquad (\Delta G°'=-112 \text{ kJ mol}^{-1}CH_4). \qquad (6)$$

Acetoclastic methanogenic archaea grow on acetate, which serves as an electron donor and acceptor:

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad (\Delta G°'=-31 \text{ kJ mol}^{-1}CH_4). \qquad (7)$$

Nevertheless, it is generally accepted that only 27-30 % of methane arises from hydrogenotrophic methanogenesis, while 70 % of methane arises from acetate.

The reduction of carbon atom in all three methanogenic pathways differs and requires different enzymes (Fig. 2; Hedderich and Whitman 2006). Only the last step, the reduction of methyl group to methane, is common for all of them and catalyzed by the key enzyme of methanogenesis – Methyl Coenzyme M reductase.

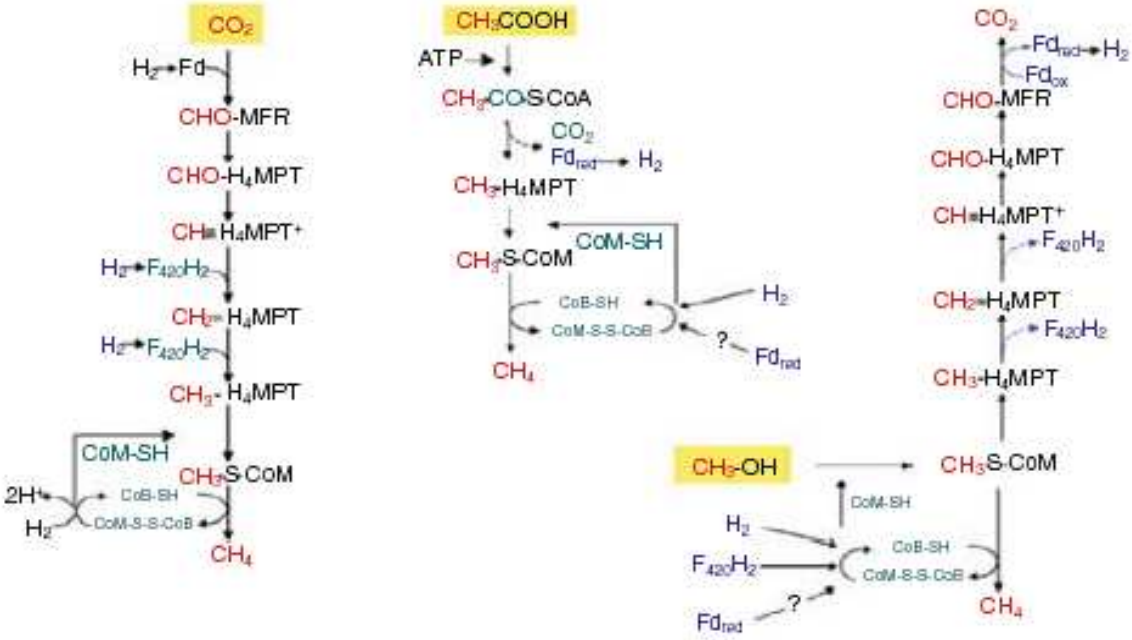

Fig. 2: *Methanogenic pathways (Hedderich and Whitman, 2006, Prokaryotes)*

1.2 Functioning of microbial communities in anaerobic digestion processes

Since anaerobic digestion is a biological process, it is imperative to understand the ecology and function of the bacterial and archaeal microbial communities and their functional guilds in those processes.

Microbiology of anaerobic digestion processes has been one of the most rapidly developing technology associated fields in the last few years. Knowledge covering the ecology and function of the microbial communities in these processes is required to better control the biological processes to maximize process yields (Fernandez et al., 1999). Therefore, considerable effort has been made to understand the microbial community structure and its correlation with system function using various molecular approaches (Plugge et al., 2002, de Bok et al., 2005, Ishii et al., 2005). Through these analyses, particularly those targeting the 16S rRNA gene, detailed pictures of the community composition are being documented. In addition, several functionally important anaerobes playing key roles in the treatment process have been cultivated and their physiological traits characterized.

Population dynamics of resident microbes in anaerobic treatment processes have also been analyzed by using rRNA and rRNA-gene based methods. For example, Shigematsu et al. (2006) studied the propionate degrading methanogenic consortium in mesophilic anaerobic chemostat using rRNA gene-based denaturing gradient gel electrophoresis (DGGE) analysis and fluorescent *in situ* hybridization (FISH). Their results indicated that dilution rates significantly affect the archaeal and bacterial communities in propionate fed chemostats. Similarly, Hori et al. (2006) analyzed changes in a thermophilic methanogenic reactor under fluctuating (deteriorative) and stable conditions, which were induced by acidification and neutralization, using rRNA gene-based single-strand conformation polymorphism (SSCP), quantitative polymerase chain reaction (PCR), and FISH. The results indicated that the original methanogenic community was significantly affected (dynamically changed) by volatile fatty acid (VFAs) concentrations in the process.

Ishii et al. (2005) used culture dependent approaches and studied interspecies hydrogen transfer between pure cultures of syntrophic bacterium *Pelotomaculum thermopropionicum* and hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus.* Results of the study revealed that *P. thermopropionicum* and *M. thermoautotrophicus* co-aggregated when they were grown in syntrophic co-culture.

Lee et al. (2008) investigated quantitative changes in the original methanogenic community in three anaerobic batch digesters treating different wastewaters, using real-time PCR based on the 16S rRNA gene and the non-metric multidimensional scaling (NMDS) analysis. Results demonstrated that the community patterns in three digesters were totally different from each other. Considering that the operating conditions in all trials were identical except for the substrates, the differences in quantitative shift profiles were suggested to be due to the different substrate compositions. This implied that the composition of wastewater could affect the evolution of quantitative methanogenic community structure in an anaerobic process. Lee et al. concluded that more attention should be directed towards the quantitative as well as qualitative changes in microbial communities for fundamental understanding of anaerobic processes, particularly under dynamic or transitional conditions.

McHugh et al. (2006) used terminal restriction fragment length polymorphism (T-RFLP) analysis of methanogenic microbial community. They monitored temporal shifts in archaeal communities during the operation of a low temperature reactor. Performance problems with reactors resulted in a decreased relative abundance of acetoclastic *Methanosaeta sp.* and an increase in relative abundance of hydrogenotrophic methanogens *Methanomicrobiales sp.*

As described in this overview, significant advances have been made in explaining the diversity of yet-to-be cultured organisms in anaerobic (methanogenic) digestion processes. Although some important research has already been published, significant advances are yet to be made in our understanding of anaerobic digestion process. The accumulation of such information will provide substantial improvement for more sophisticated management of the anaerobic digestion technology. Such analyses could also find application in the study of anaerobic digestion inhibition by the accumulation of trace elements or toxic compounds, caused by application of less degradable substrates to the microbial biomass.

2 AIMS, OBJECTIVES AND THESIS STRUCTURE

The aim of this work was to establish a reliable protocol for bio-monitoring process of anaerobic degradation coupling the conventional parameters (temperature, pH, VFA, hydrogen partial pressure, CH_4 and CO_2 gas composition) and molecular approaches (PCR, T-RFLP, cloning and sequencing, microarray) with the adaptive changes in the methanogenic microbial community in response to environmental perturbation (e.g. new substrate). As a model of adaptation to the new substrates we selected different substrates: readily degradable substrate (brewery waste water) was used for the optimization of BMP assay whereas less degradable substrates (brewery spent grain, cyanide) were used in the subsequent experiments. The effect of different concentrations of substrates on microbial community structure and on methanogenesis was determined by correlating adaptivity of microbial community structure to the parameters of methane production (yield, dynamics, etc).

We identified the following four objectives of our work:

- To establish methodologies for bio-monitoring process of anaerobic degradation by selecting and evaluating suitable experimental methods to be used throughout the study;
- To estimate the effect of less degradable substrate (brewery spent grain, cyanide) on bacterial and archaeal microbial communities;
- To determine the possibility of cyanide degradation in two distinct microbial biomass samples in relation to previous findings (Gijzen et al., 2000, Fallon et al., 1991, Fedorak and Hrudey 1989);
- Identify the key microbial constituents in the microbial biomass samples involved in cyanide degradation.

Each of these objectives is described in its own chapter as follows:

CHAPTER 1: Optimization of BMP assay
CHAPTER 2: General microbial community flexibility in biochemical methane potential assay is highly correlated to initial biogas production rates
CHAPTER 3: Cyanide degradation in two contrasting anaerobic digester biomass samples (five experiments)
CHAPTER 4: Microbial communities in cyanide degradation experiments
CHAPTER 5: Thesis discussion and conclusions

First, we established a method for predicting the effectiveness of available methanogenic biomasses in the event of substrate change. Acceptably adaptive seeding biomass can be identified by the use of molecular tools complemented by functional performance to ensure quicker and more economical bioreactor start up for different substrates. Therefore, with the use of molecular techniques in combination with already known methods for testing methane production, we established bio-monitoring procedure, which can be further used for planning a stable anaerobic methanogenic process and also for routine monitoring of its stability. Results are presented and discussed in Chapter 1.

Secondly, we preformed biochemical methane potential (BMP) assays with spent brewery grain as substrate that has not been treated by any of the three test biomasses used in experiments in order to determine the most suitable microbial biomass for effective start-up of anaerobic bioreactor. Results are presented and discussed in Chapter 2.

Thirdly, BMP assay was performed with wastewaters containing cyanide in different concentrations (Experiments 1-5). Two distinct biomasses with completely different physiology and process history were used in these experiments (Experiments 1-3 and Experiments 4-5, respectively). Their adaptation capabilities to cyanide loading were tested as was described before (Gijzen et al., 2000, Fallon et al., 1991, Fedorak and Hrudey 1989).

At last, microbial communities in the above described experiments were explored in order to identify the key players responsible for apparently biotic cyanide degradation. The transition of microbial community to cyanide degrading consortia was described. In addition, cloning and sequencing determined the detailed microbial community structure of bacterial and archaeal microbial communities. In the experiments where changes in microbial community structure were detected microarray analyses of methanogenic archaea was performed (Chapter 4). Each section contains a focused introduction, material and methods section, result presentation and detailed discussion of the outcomes of the respective experiments. The discussion and conclusions of the entire thesis are presented in Chapter 5.

CHAPTER 1

CHAPTER 1 – OPTIMIZATION OF BMP ASSAY

1 INTRODUCTION

Batch assays such as the Biochemical Methane Potential (BMP) or the anaerobic toxicity assays (ATA) (Owen et al., 1979) have been routinely used to determine optimal reaction conditions or compound toxicity due to the level of experimental control they offer in bench top or pilot plant experiments (Jessup et al., 2004; Drake et al., 1996; Owen et al., 1979). Many variations of the BMP assay are available commercially. The relatively inexpensive Oxitop® (WTW, Germany), which is also frequently used for biological oxygen demand (BOD) measurements (Voorthuizen et al., 2008, Fan et al., 2009 Bouzas et al., 2007) was adapted and optimized for our needs. OxiTop® has technical limitations, since the pressure increase due to biogas formation is detected only up to 350 hectopascals (hPa). Therefore, loading rates must be calculated carefully. During the period of incubation pressure increase must be carefully monitored and if necessary it can be released without changes in the composition of biogas during the period of incubation. We tested our general experimental set-up with a microbial biomass from industrial UASB reactor and substrates, at mesophilic conditions (37°C) to establish a system for monitoring the degradation process effectiveness, community dynamics and structure-function relationship of methanogenic biomass in the event of substrate change.

Research work described in this section was directed primarily to BMP assay optimization with the system OxiTop® (e.g. biogas production, sampling, VFA extraction and separation, biogas composition) and terminal restriction fragment length polymorphism (T-RFLP) technique optimization for the high-through put analysis of microbial communities of our microbial biomass samples (all steps involved e.g. sampling, sample manipulation, preparation and storage, DNA extraction, PCR, selection of restriction enzymes, cloning and sequencing).

2 MATERIALS AND METHODS

2.1 Optimization of BMP assay, sampling and analyses

The Oxitop® (WTW, Germany) consists of 1000 mL glass bottles with measuring heads (BMP bottles) that record the gas pressure changes in the bottles (Fig. 3). The system simulates a batch anaerobic bioreactor. The BMP assay was used to measure biogas production during the anaerobic digestion process. A microbial biomass served as a source of microorganisms degrading organic matter of the substrate samples. The biogas produced increased the bottle pressure and thus served as a general indicator of the extent and rate of production (measuring head record pressure in hPa). Basal biogas production of biomass (blank samples) representing a negative control test was monitored in parallel with the test substrate bottles. According to test specifications, biomass was loaded with appropriate amounts of substrate to ensure measurable range of biogas production throughout the experiment.

Before the set up of the experiments it was necessary to obtain information on total solids (TS) and volatile solids (VS) of the microbial biomass first. In addition to these data, it was necessary to obtain further information on the total organic carbon (TOC) or chemical oxygen demand (COD) of the substrate in order to calculate the prerequisite biomass/substrate ratio needed for the correct set up of starting mixtures in the experimental bottles.

The specified amount of microbial biomass was preincubated at 37°C for 5 days according to the standard protocol (APHA, 2005) before substrate, water, buffer and nutrients was added to the microbial biomass. In order to ensure anaerobic conditions, bottles were purged with nitrogen gas for 10 min before being sealed and thermostated at 37°C (mesophilic conditions) for at least 21 days. The contents of the bottles were constantly mixed at 120 rpm throughout the experiment.

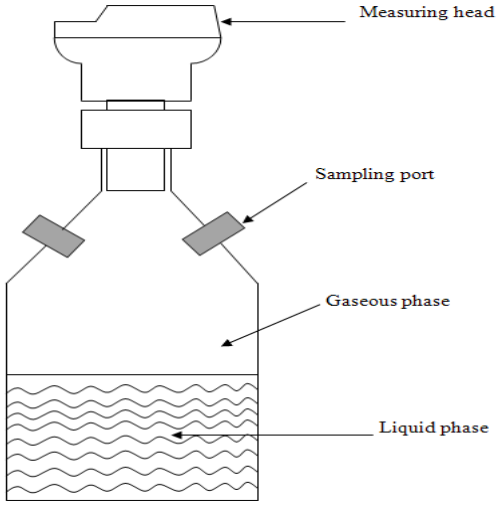

Fig. 3: *Schematic view of Oxitop bottle*

The Oxytop® bottles allow sampling of ~~a~~ the liquid or the gas phase during the course of the test with syringe and injection needle through the butyl rubber septum covering the sampling port, which is located on the side neck of the bottle and prevents the entry of air into the bottle during sampling (sampling port on Fig. 3). Since this is a batch system with a capacity of 1000 mL, sampling of small volumes should not disturb the process, therefore the frequency of sampling, and cumulative volumes of the removed analytical samples had to be adjusted to represent less than 5-10% of the initial total liquid and gas volume. To control sampling and test the logistic requirements, the tests needed to be assembled in replicates (up to 9) so that at least three bottles for each individual treatment remained at the end of the experiment.

During the test, liquid and gas samples were taken from each bottle separately in most informative time intervals, which were determined on the basis of preliminary results and recommendations in the published literature. Biogas composition, i.e. the percentage of CH_4, CO_2 and H_2, was followed with gas chromatography using a Shimadzu (Japan) gas chromatograph (GC) with the thermal conductivity detector (TCD). For GC analysis of biogas composition only 20-50 µl of gas phase are required thus avoiding significant variations in the volume of biogas inside of test bottle. The gases were separated using 4 m long steel column with inner diameter of ¼ inch filled with Porapak Q. Helium was used as ~~a~~ the carrier gas. The temperatures of the injection port and of the column were 30° C, and the detector temperature was

80° C. Calibration gas mixtures contained hydrogen, nitrogen, methane and carbon dioxide. Chromatographic signals were evaluated by integrator Chromatopack CR-4A (Shimadzu) based on an absolute calibration. Integration of individual peaks was made by OriginPro 8th edition (Originlab, USA). VFAs were analyzed after double ether extraction with gas chromatography on Schimadzu GC-14A with split/splitless injector and flame ionization detector (FID).

During the test, the rising pressure in the bottles was recorded in one-hour intervals by measuring the heads (Oxitop pressure recorder), reflecting the production of biogas from degradation of organic substrate tested. After the test, the data collected were transferred to the computer with a control module. In order to express the quantity of biogas, which occurs only at the expense of added substrate, measurements of blank sample were subtracted from the measurement of the test mixtures. The differences were converted into the volumes of biogas using general gas law and were graphically presented as the cumulative production of biogas. From the fraction of CH_4 (%) in the biogas and from the total volume of biogas in the relevant time intervals we similarly calculated the cumulative production of methane.

$$\frac{P_0 \cdot T_0}{T_0} = \frac{P_x \cdot V_x}{T_x} \qquad (8)$$

P_0 - pressure at standard conditions (101,3 kPa)

V_0 - volume of gas at standard conditions

T_0 - temperature at standard conditions (273 K)

P_x - measured pressure

V_x - available volume in the bottle (V_{bottle} -$V_{mixture}$ = 710 mL)

T_x - temperature of incubation (310 K)

In a well-performed methanogenic anaerobic degradation, methane is the dominant gas in the biogas (60-80 %) whereas hydrogen typically represents far less than 1 %.

2.2 Optimization of microbial community structure analyses

The structure of microbial communities (bacterial and archaeal) was analyzed at several time periods. In this way we were able to track possible cyclic changes or responses of microbial communities to the given substrate, or a mixture of substrates and the effects of incubation in the experiment. Due to the slow growth of anaerobic bacteria and methanogenic archaea, it was expected that adaptation of microbial community to new environment would also be slow but detectable within the time span of experiments (tested at least at the beginning and at the end of BMP assay).

For the analysis of the structure of methanogenic communities a technique termed terminal restriction fragment length polymorphism (T-RFLP) based on 16S rRNA gene was used. T-RFLP is a molecular biology technique of high phylogenetic resolution, suitable for high through put and cost-effective profiling of microbial communities based on the position of a restriction site closest to a labeled end of the amplified gene. Thus, numerous samples representing microbial community biomass can be screened and community changes identified. The method is based on digesting a mixture of PCR amplified variants of a single gene using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer (Fig. 4; Grüntzig et al., 2002).

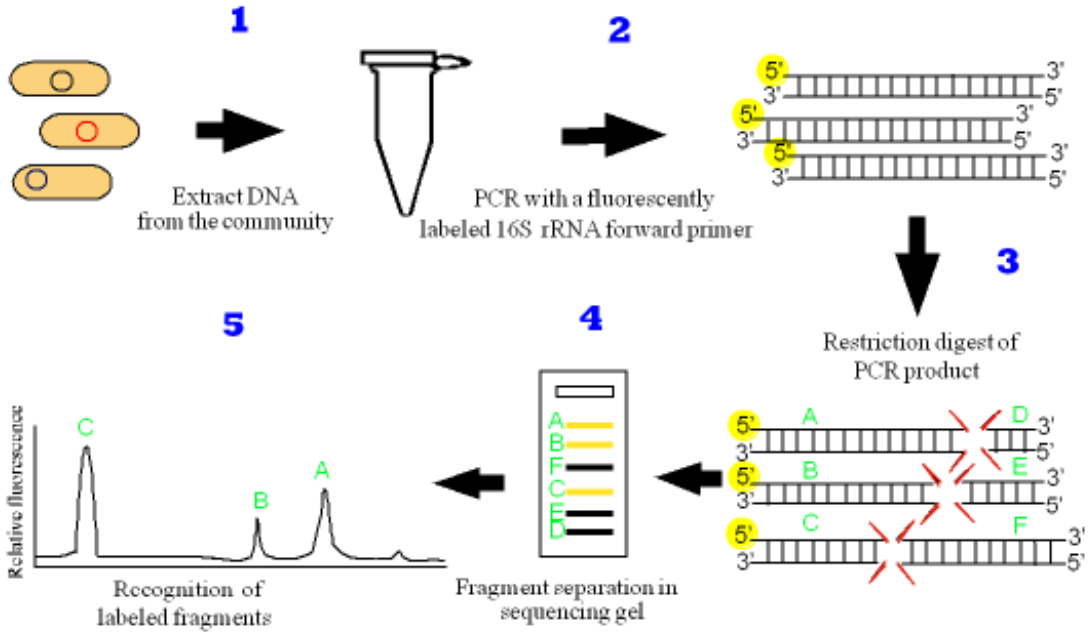

Fig. 4: *Illustration of T-RFLP method (Grüntzig et al., 2002)*

The result is the image where the X-axis represents the sizes of the fragment and the Y-axis represents their fluorescence intensity (Liu et al., 1997). We chose to use the most frequently used restriction enzymes; *HhaI*, *MspI*, *HaeIII* and *HindIII* (Kitts et al. 2001, Osborne et al., 2000).

Comparison of T-RFLP profiles (presence / absence of peaks and their intensity of fluorescence) was performed using Bio Numerics 5.1 software (Applied Math, Belgium). Similarity calculations of different T-RFLP profiles were based upon densiometric curves and for calculation of similarity matrix the Pearson correlation was used. Relationship between samples was visualized by grouping them in a dendrogram using unweighted pair-group method with arithmetic mean (UPGMA) clustering method. UPGMA is a pairwise clustering algorithm that uses similarity matrix as input for construction of a rooted tree (dendrogram) and is one of most commonly used methods for similarity-based clustering.

2.3 Reactor operation, inoculums source and characteristics

In total 35 Oxitop bottles (WTW, Germany) with working volumes of 1000 mL were used as anaerobic batch reactors. Oxitop bottles were equipped with pressure sensors. They were operated anaerobically, thermostated at 37°C (WTW, Germany), and protected from light for 21 days. Side necks (Sampling ports on Fig. 3) were sealed with new butyl rubber stoppers (WTW, Germany).

Reactors were inoculated with 43 mL (according to VS of microbial biomass) of anaerobic granulated biomass obtained from an industrial upflow anaerobic sludge blanket (UASB) reactor. The UASB reactor was operating more than 2 years at 37°C and was loaded with brewery wastewaters at a loading rate 4g COD/L/day and hydraulic retention time of 40 hours. The soluble organic carbon removal efficiency measured as COD was greater than 90 %.

Regarding to the COD of the substrate (Table 1), biomass was loaded with 50 mL and 62 mL brewery wastewater (BWW) from the same UASB reactor supplemented with 0.37% of fresh (FBY) or hydrolyzed brewery yeast (HBY), respectively. COD

was 4095 ± 113 mg/L and 3.127 ± 123 mg/L for HBY and FBY, respectively. Loading rate was 0.2 gCOD/$gVS_{biomass}$. Phosphate buffer (20 mL) and diluted fresh deoxygenated water was added to the 500 mL mark. Each substrate was run in 7 parallels together with additional blank sample for negative control and sample for positive control with added glucose (loading rate was 0.2 gCOD/$gVS_{biomass}$).

2.4 Analytical techniques and assays

Gas composition was measured on days 1, 3, 6, 10, 14, 21 with gas chromatograph (Shimadzu, GC14A-TCD). The gases were separated using 4 m long steel column with inner diameter of ¼ inches, filled with Porapak Q. Helium was used as a carrier gas. Injector and column temperature was 30° C and the detector temperature was 80° C. Calibration gas mixtures contained hydrogen, nitrogen, methane and carbon dioxide. Chromatographic signals were evaluated by integrator Chromatopack CR-4A (Shimadzu) based on an absolute calibration.

The concentration of VFAs was determined on days 0, 3, 6, 10, 14, 21 using gas chromatograph Schimadzu GC-14A with split/splitless injector and flame ionization detector (FID), equipped with fused silica capillary column DB-WAX of 30 m length, 0.326 mm diameter and 0.25 μm stationary phase thickness (J&W Scientific, 123-7032). Quantification was performed by an internal standard method. The injector temperature was 160 °C, the detector temperature 210 °C, the column temperature 75 °C. Volatile fatty acids (VFAs C1-C5 molecules) were extracted from the samples with diethyl ether according to the procedure of Holdeman et al. (1977) and analysed.

The efficiency of soluble organic carbon removal was monitored by analyzing COD, which was measured by LCK 514 COD cuvette test (Hach-Lange, Germany) according to the manufacturer instructions. The principle of determination is that oxidizable substances react with sulphuric acid – potassium dichromate solution in the presence of silver sulphate as a catalyst. Chloride is masked by mercury sulphate. The green coloration of Cr^{3+} is evaluated photometrically using Hach-Lange DR2800 VIS spectrophotometer at 605 nm.

2.5 DNA extraction, PCR amplification of partial 16S rRNA genes and T-RFLP analysis

The genomic DNA was extracted from the microbial biomass samples using UltraClean Soil DNA Isolation kit (MO BIO, USA) according to the manufacturer's instructions. The quality of DNA was checked by separation in 1% agarose (SeaKem, USA) gel electrophoresis (BioRad, USA). Extracted DNA was purified with High Pure PCR purification kit (Roche, Switzerland) and then used as PCR template. PCR amplification was performed using MyCycler (BIO-RAD, USA). For amplification of 16S rRNA gene of methanogenic archaea 109f (5' - CAN GCT CAG TAA CRC GYR-3') and 691r (5'-CGA TTA CAR GAT TTC AC-3') PCR primers were used (Watanabe et al., 2004). Bacterial 16S rRNA genes were amplified using 27f (5' - AGA GTT TGA TCC TGG CTC AG-3') and 926r (5'-CCG TCA ATT CCT TTR AGT TT-3') primer pair. For T-RFLP analysis, forward primer was labeled with 6-FAM on a 5'-end. The bacterial and archaeal PCR started with 5 minutes initial denaturation at 94°C. A total of 32 cycles included 60 s at 94°C, 60 s at 53°C, and 60 s at 72°C, and was followed by a final extension step of 10 min at 72°C. The correct length of PCR products was examined with electrophoresis on 1 % agarose gels. PCR products were digested with *Hae*III restriction enzyme (Fermentas Inc, USA) over night at 37°C and deactivated at 80°C for 20 min. prior to T-RFLP analysis, salts and inactivated enzymes were removed with ethanol precipitation (Ausubel et al., 1999).

After precipitation, 2 µL of each sample was mixed with 2 µL of DNA internal standard (TAMRA 1000 fragment length standard, PE Biosystems, USA) and 2 µL of formamide and 0.5 µL loading buffer. Prior to fragment analysis, samples were denatured at 94°C for 5 min and then chilled on ice for 5 min. A fragment size analysis was carried out with an ABI 3100 genetic analyzer (Applied Biosystems, USA). Only terminal restriction fragments (TRFs) with total fluorescence exceeding the limit of 10.000 units were analyzed. The relative abundance of a TRF in a T-RFLP profile was calculated by dividing the peak height of the TRF by the total peak height of all TRFs in the profile. All peaks with heights that were less than 0.5% of the total peak height were not included in further analyses. This approach minimized

the effect of variations in the T-RFLP profiles caused by the quantity of DNA analyzed.

T-RFLP profiles were analyzed with Bio Numerics (Applied Maths, Belgium) and comparisons were presented as dendrograms.

3 RESULTS AND DISCUSSION

3. 1 Batch reactors performance

Three different substrates (Table 1) were added to the inoculum - anaerobic granulated biomass obtained from industrial UASB reactor at 0.2 g/COD of substrate to the 1 g/VS of microbial biomass. Besides substrates, a blank control was used (e.g. biomass without substrate for negative control) and glucose as a substrate for positive control. The initial substrate characterizations are presented in Table 1.

Table 1: *initial substrate characterizations (Values are means for 3 replicates, variance did not exceed 5 %)*

	Biomass	Fresh yeast	Hydrolyzed yeast	Substrate 1 Brewery waste water (**BWW**)	Substrate 2 Mixture BWW+0,37% **H**ydrolyzed **B**rewery **Y**east (**HBY**)	Substrate 3 Mixture BWW+0,37% **F**resh **B**rewery **Y**east (**FBY**)
TS	8.95 g/L	152 g/L	186 g/L	9.89 g/L	9.76 g/L	9.76 g/L
VS	23.07 g/L	142 g/L	177 g/L	1085 g/L	1.41 g/L	1.26 g/L
TOC	/	76.07 g/L	49.99 g/L	0.728 g /L	1100 g/L	0.79 g/L
COD	/	293 g/L	277 g/L	3062 g/L	4095 g/L	3172 g/l

The highest VS content was observed in HBY. Content of VS in BWW was quite similar. The same pattern was observed in the measurements of COD and TOC.

The process was successfully completed and high cumulative biogas production was recorded (Fig. 5, Fig. 6, Fig. 7). Most of the biogas was produced during the first 6

days. However, the average biogas production rate for BWW was 3.24 ± 0.3 mL/day, for HBY 2.91 ± 0.42 mL/day and for FBY 3.81 ± 0.32 mL/day.

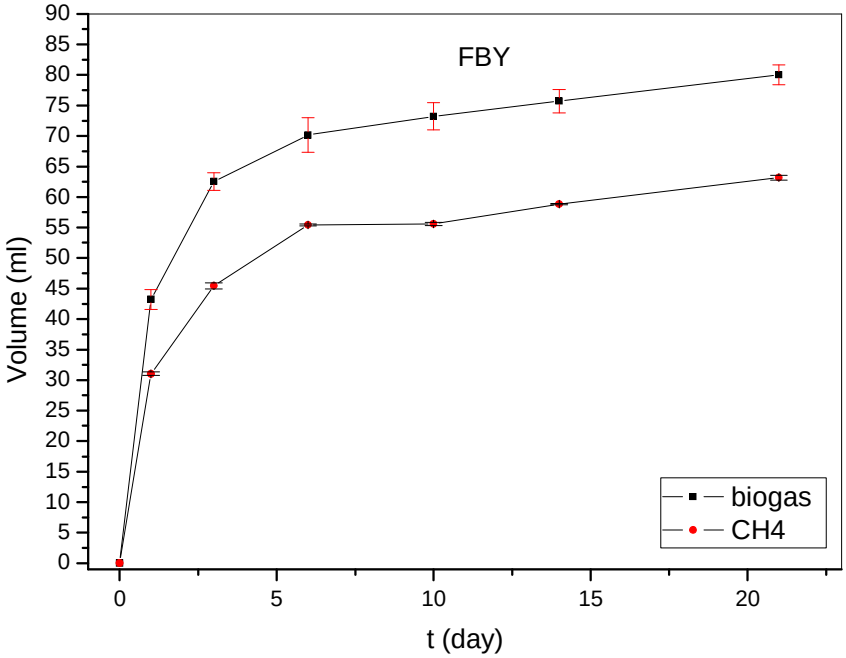

Fig. 5: *Cumulative biogas and methane production for Fresh Brewery Yeast and Water (FBY) (loading rate 0.2gCOD/gVS$_{biomass}$).Values are means of three replicates ± SD.*

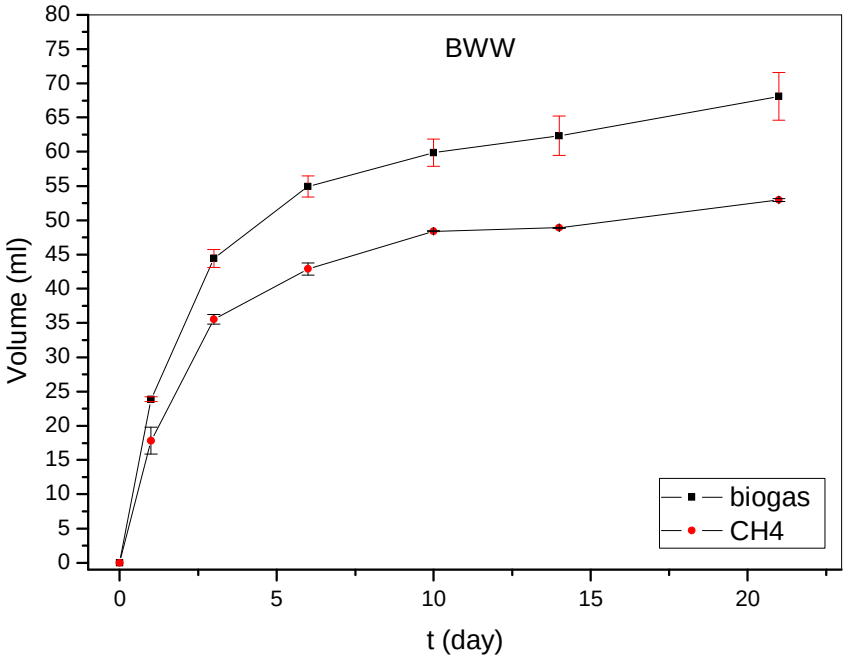

Fig. 6: *Cumulative biogas and methane production for Brewery Waste Water (BWW) (loading rate 0.2gCOD/gVSbiomass).Values are means of three replicates ± SD.*

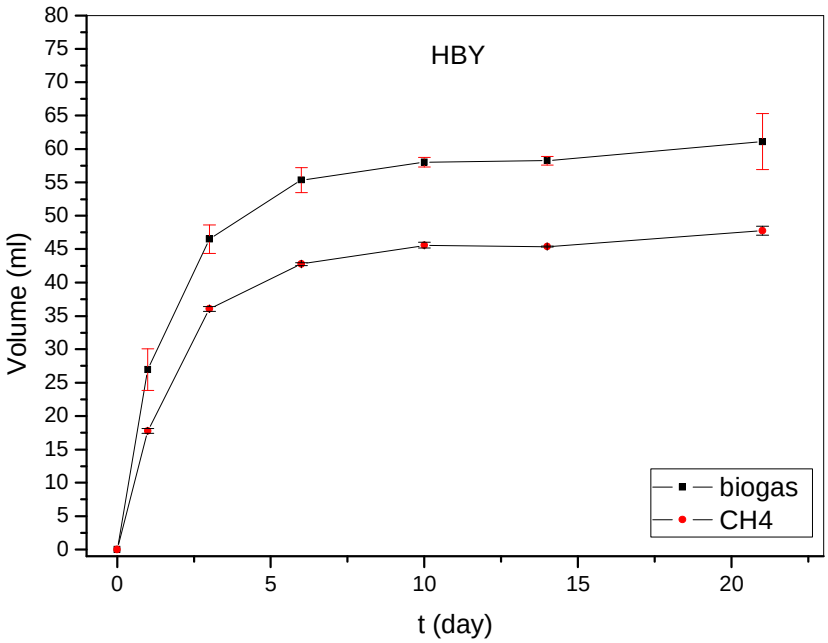

***Fig. 7**: Cumulative biogas and methane production for Hydrolized Brewery Yeast and Water (HBY) (loading rate 0.2gCOD/gVS$_{biomass}$). Values are means of three replicates ± SD.*

The highest biogas production was observed in the bottle supplemented with FBY as a substrate (Fig. 5, Fig. 8) and the lowest biogas production was in the bottle that contained HBY (Fig. 7, Fig. 8)

After 6 days of operation, most of the methane was produced (Fig. 8). The results showed that the addition of FBY resulted in higher production of methane.

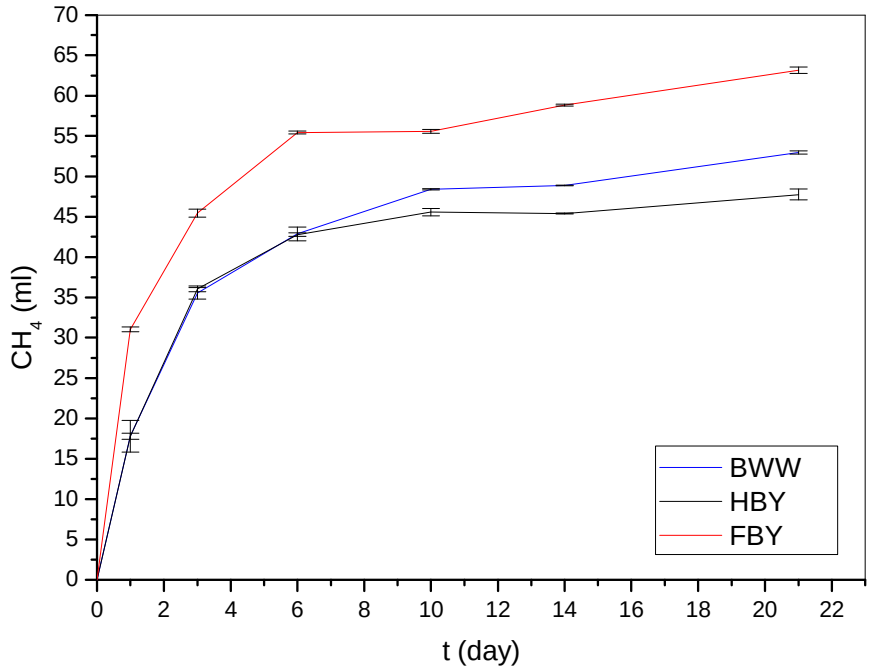

Fig. 8: *Cumulative methane production (loading rate 0.2gCOD/gVS$_{biomass}$). Values are means of three replicates ± SD.*

Maximum cumulative methane production was detected in reactor containing FBY as substrate. At a loading rate 0.2gCOD/gVS cumulative (21 days) production of methane was 63.17 ± 0.9 mL CH_4 and 45.75 ± 1.2 mL CH_4 for FBY and HBY material, respectively.

We also calculated methane yields as the ratio between the theoretical values of methane production from 1 kg degraded COD and actual production of methane from 1 kg of degraded COD in the BMP test according to the standard (ISO 11734:1995). Theoretical value assumes that from 1 kg of degraded COD gives 0.35 m^3 of methane at standard conditions; an approach routinely used in industrial wastewater treatment plants.

The calculations revealed that methane yield for BWW was 75.6 %, for HBY 65.7 % and for FBY 91.5 %.

Although pH values decreased slightly in all of the reactors during the incubation (from 7.08 to 6.94 (Fig. 9), the values were still in the optimum area and did not differ significantly. The pH optimum of the methane production in anaerobic bioreactor is at pH = 6.7-7.5 (Deublein et al., 2008).

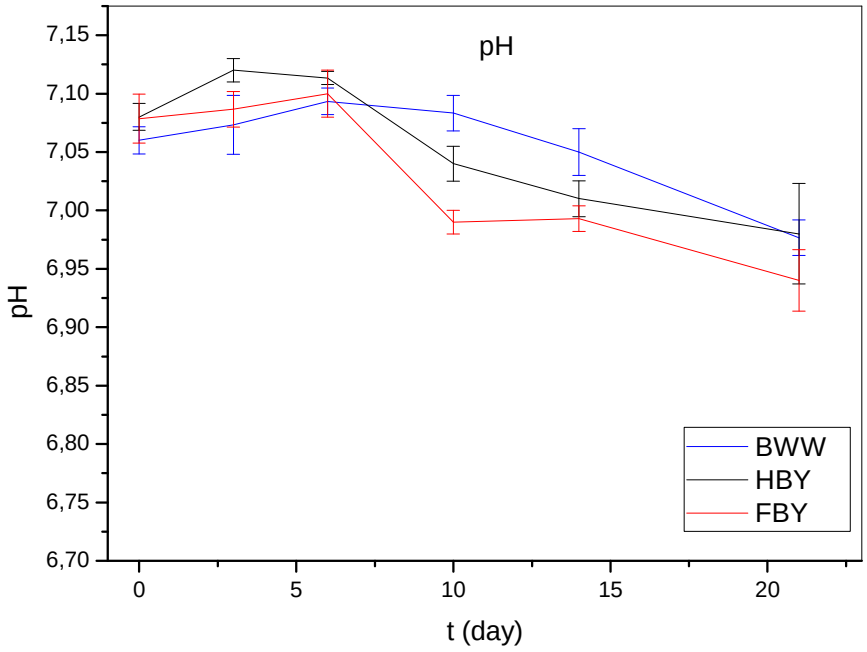

***Fig. 9**: pH value during the experiment. Values are means of three replicates ± SD.*

Acetate and propionate were the major VFAs formed initially. After 3 days of operation, VFAs concentrations decreased (Fig. 10) and were completely degraded at day 10, with the exception of acetic acid.

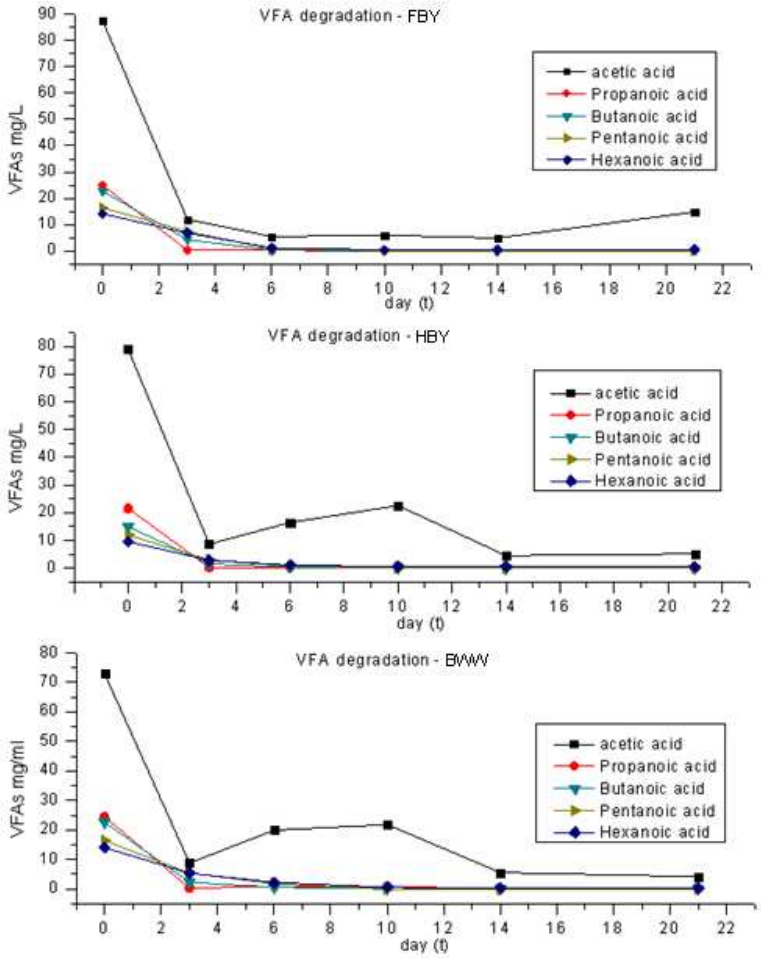

Fig. 10: *Concentrations of VFAs. Values are means of three replicates; SD is omitted for clarity but represented less than 10% of the average.*

In the case of brewery wastewater (BWW) and hydrolyzed yeast (HBY), acetic acid started to accumulate again at day 3 and was mostly degraded by day 14. However, this did not affect the methane production (Fig. 11). Acetic and propionic acid concentrations were highest at the beginning of the process as was described before (Chen et al., 2008).

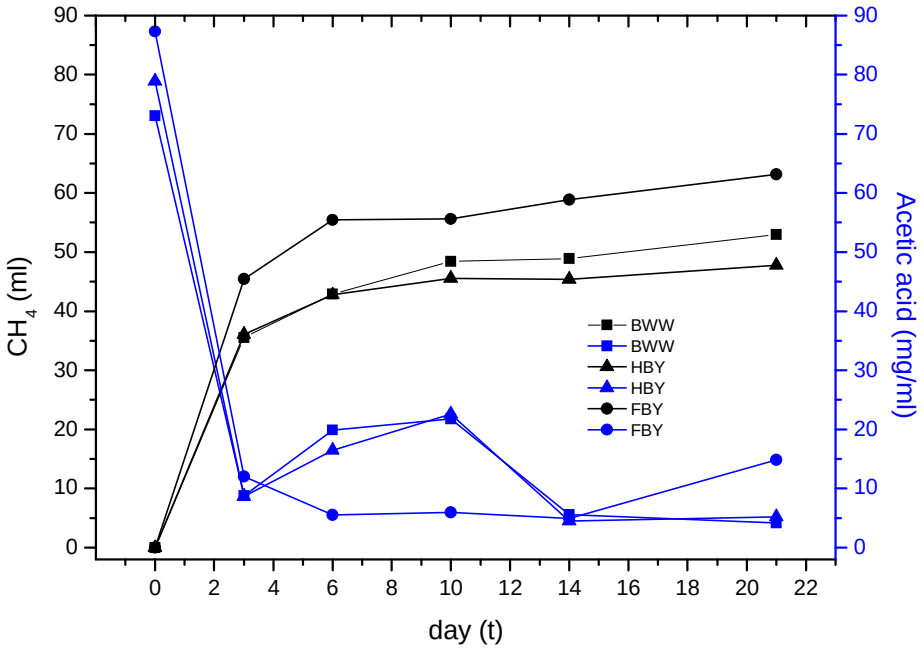

Fig. 11: *Accumulation of methane (CH4) and acetic acid concentrations. Values are mean of three replicates; SD is omitted for clarity but represented less than 10 % of the average.*

3.3 Microbial community structure analysis

Biomass samples were collected directly from Oxitop bottles. Bottles were sampled six times, at days 0, 1, 3, 6, 10, 14, 21, during 21 days of operation. Each of the samples was run in three parallels. To explore the overall variability in microbial community structure, DNA was extracted from day 0 (t_0), day 6 (t_6) and day 21 (t_{21}) samples. The quality of DNA was checked with agarose gel electrophoresis (Fig. 12).

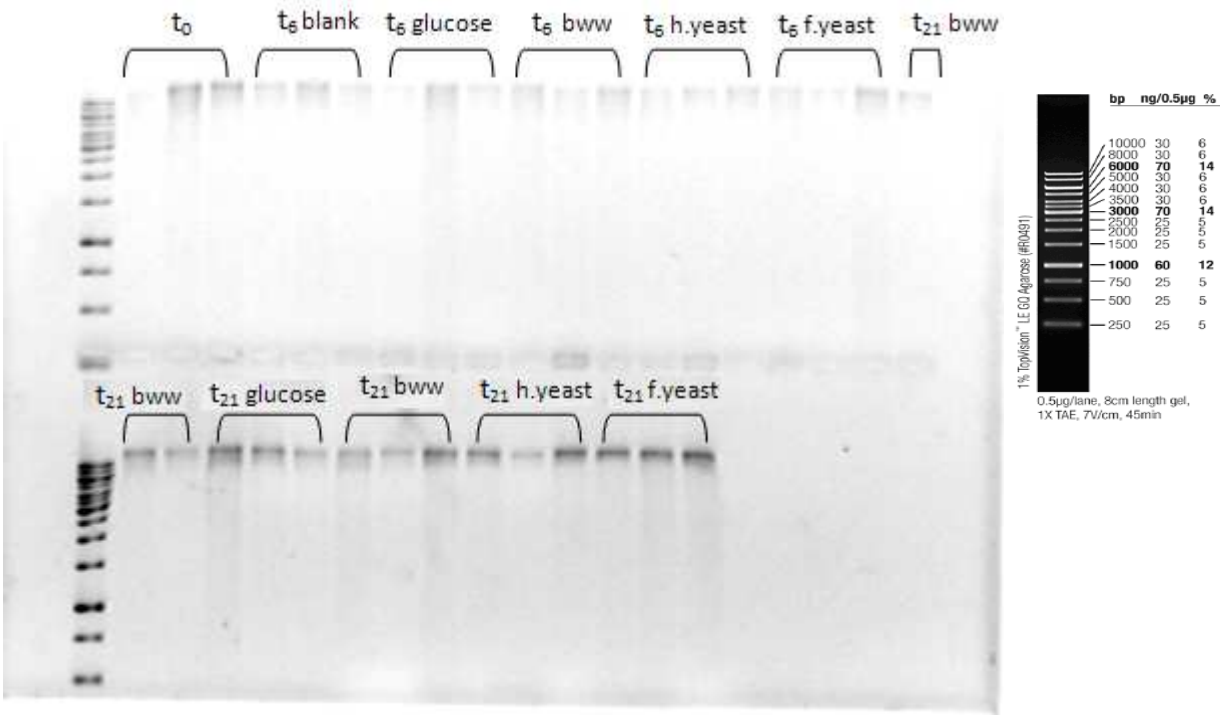

Fig. 12: *Agarose gel electrophoresis of DNA extracts (f.yeast- FBY, h.yeast - HBY, BWW-brewery waste water, blank - negative control, glucose - positive control) and corresponding DNA ladder.*

The extracted DNA was stored at -20°C, and used as template for archaeal (Fig. 13) and bacterial (Fig. 14) polymerase chain reaction (PCR) amplification.

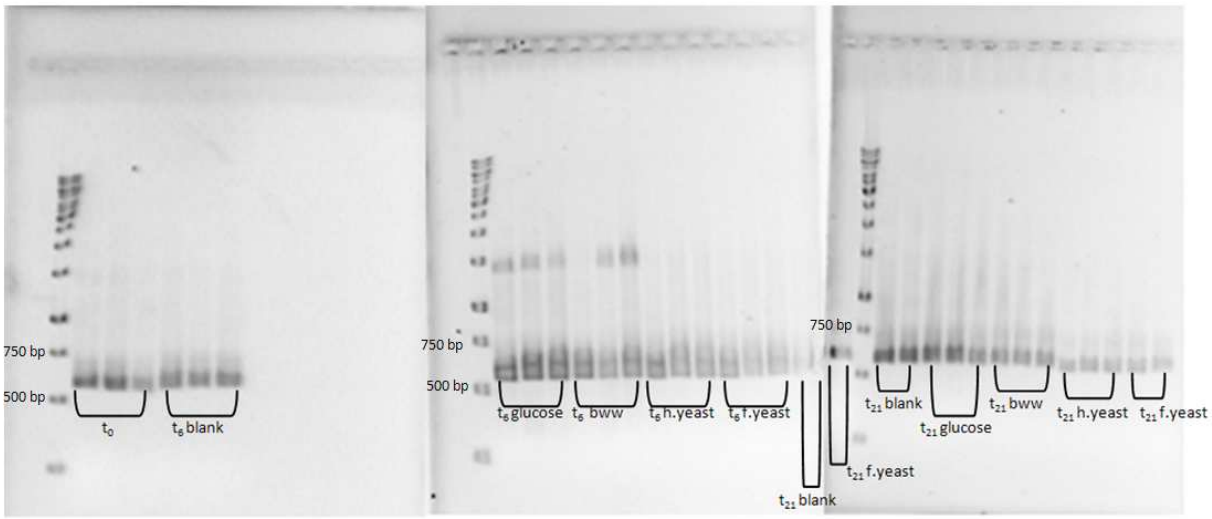

Fig. 13: *Agarose gel electrophoresis of archeal PCR products (f.yeast- FBY, h.yeast - HBY, BWW-brewery waste water, blank - negative control, glucose - positive control) and DNA ladder on the left side of the gel with indicated length of DNA fragments (t0 – day 0, t6 – day 6, t21 – day 21).*

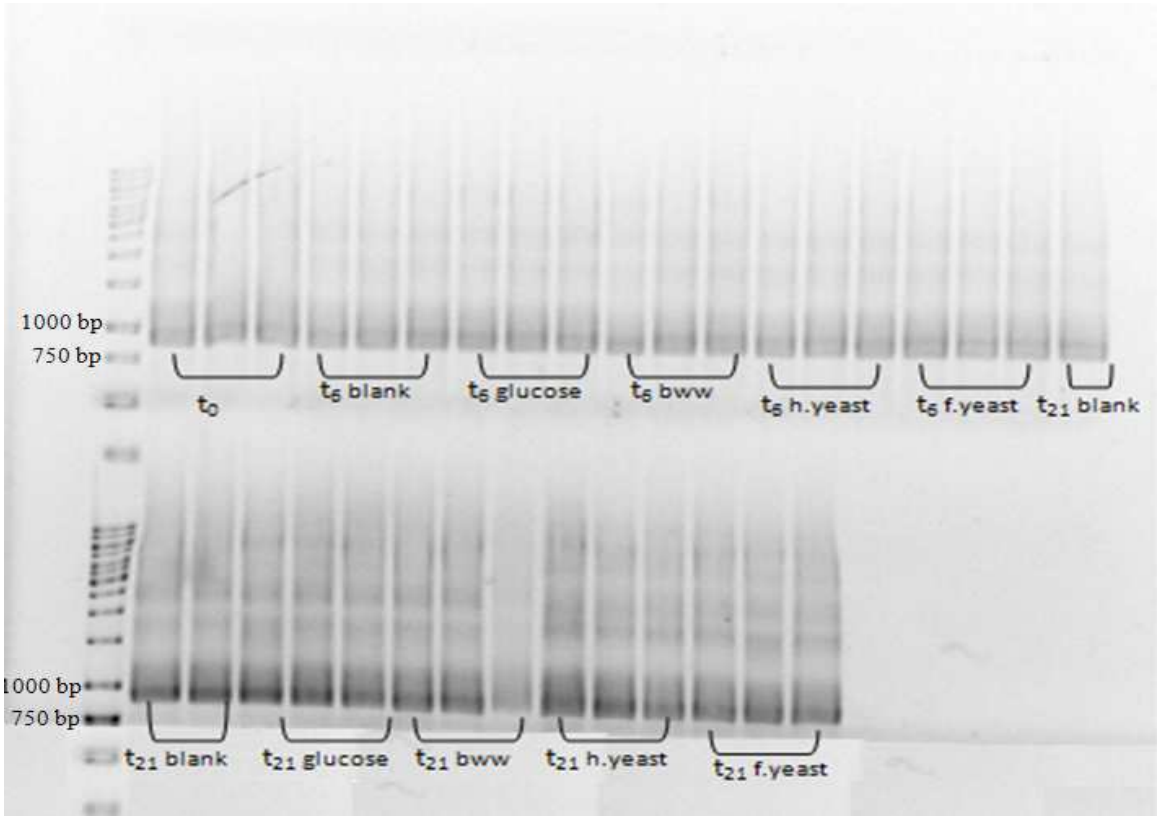

Fig. 14: *Agarose gel electrophoresis of bacterial PCR products (f.yeast- FBY, h.yeast - HBY, BWW-brewery waste water, blank - negative control, glucose - positive control) and DNA ladder on the left side of the gel with indicated length of DNA fragments (t0 – day 0, t6 – day 6, t21 – day 21).*

PCR amplification was successful and restriction reactions were performed. After enzyme deactivation, fragment size analysis was carried out on an automatic sequencer. Resulting T-RFLP profiles were analyzed and hierarchically clustered based on Pearson correlation, and dendrogram was constructed using UPGMA method (Fig. 15 and Fig. 16).

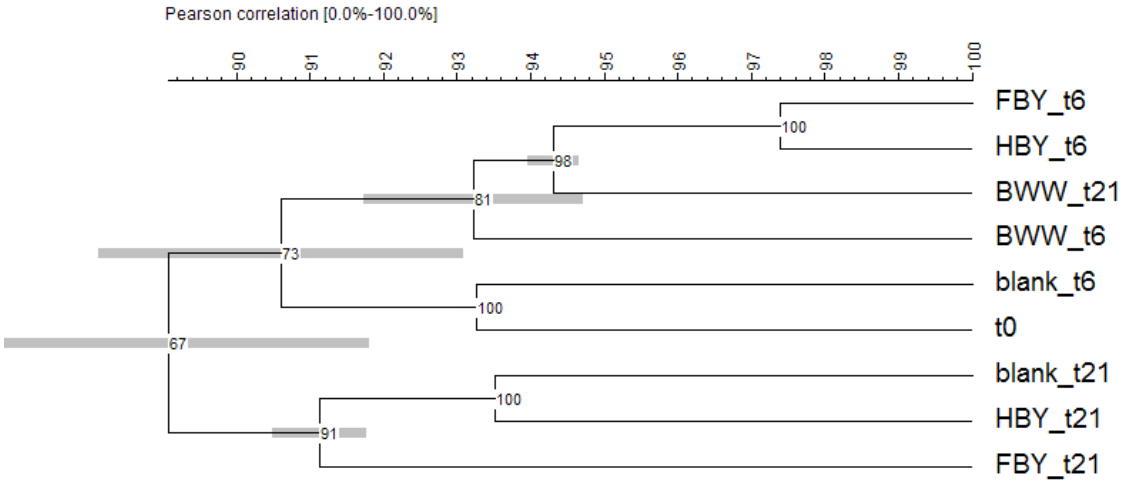

Fig. 15: *Pearson correlation dendrogram of T-RFLP fingerprints of methanogenic microbial communities (t0 – initial state, FBY_t6 f.yeast at day 6, FBY_t21 f.yeast at day 21, HBY_t6 h.yeast at day 6, HBY_t21 h.yeast at day 21 BWW_t6 brewery waste water at day 6, BWW_t21 brewery waste water at day 21, blank_t6 - negative control at day 6, blank_t21 - negative control at day 21). Gray lines delineate relevant clusters separated by the cluster-cut off method and similarity values (cophenetic correlation) are shown as numbers at each branch. Horizontal bars (error flags) represent the reliability and internal consistency of the branch.*

The average similarity and the exact standard deviation are shown at the position of the cursor. The smaller the horizontal bar, the more consistent a group is. The cophenetic correlation is also shown at each branch (number) and this is a parameter to express the consistency of a cluster. This method calculates the correlation between the dendrogram-derived similarities and the matrix similarities (BioNumerics manual, AppliedMaths, Belgium).

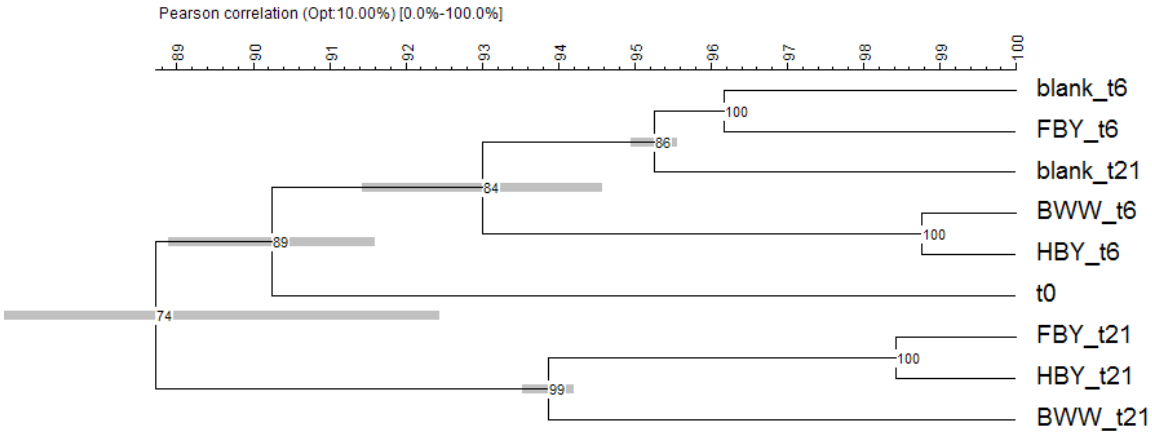

Fig. 16: *Pearson correlation dendrogram of T-RFLP fingerprints of bacterial microbial communities (t0 – initial state, FBY_t6 f.yeast at day 6, FBY_t21 f.yeast at day 21, HBY_t6 h.yeast at day 6, HBY_t21 h.yeast at day 21 BWW_t6 brewery waste water at day 6, BWW_t21 brewery waste water at day 21, blank_t6 - negative control at day 6, blank_t21 - negative control at day 21). Horizontal gray lines delineate relevant clusters separated by the cluster-cut off method and similarity values (cophenetic correlation) are shown as numbers at each branch. Horizontal bars (error flags) represent the reliability and internal consistency of the branch.*

Multivariate statistical analyses showed that there was no significant change either in bacterial (only 12 % dissimilarity) or archaeal (only 11 % dissimilarity) microbial community structure during 21 days of anaerobic degradation in response to either yeast material as substrates (Fig. 15, Fig. 16). Such results were expected since the substrates were mainly brewery wastewater supplemented with very low amount of yeast materials (0.37 %) and granulated biomass originated from UASB reactor treating the same brewery wastewater as before.

Tested substrates were used (i) for optimization of the BMP test, (ii) to elucidate the effects of minor additions of novel substrates on system functioning, and (iii) to optimize T-RFLP analysis for bacterial (hydrolysis, acidogenic, acetogenic) and archaeal (methanogenic) consortia. We successfully simulated batch anaerobic reactor and degradation processes. We were able to monitor anaerobic digestion parameters (biogas production and composition, VFAs, pH, temperature). We were also successful at DNA extraction, PCR and microbial community structure analysis.

3.4 Conclusions

We used the system (Oxitop) and optimized the system for our needs. A manageable system for monitoring the degradation process effectiveness, community dynamics and structure-function relationship of methanogenic and also bacterial biomass in the event of substrate change was established. In this experiment, the addition of limited amounts of different substrates resulted in significantly different system functioning; however, the underlying microbial communities remained stable and unaffected.

CHAPTER 2

Published in:

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CHAPTER 2 – GENERAL MICROBIAL COMMUNITY FLEXIBILITY IN BIOCHEMICAL METHANE POTENTIAL ASSAY IS HIGHLY CORRELATED TO INITIAL BIOGAS PRODUCTION RATES

1 INTRODUCTION

Monitoring anaerobic digestion is a difficult and complex multivariate process (Ward et al., 2008). Performance of such systems can be related to a number of different environmental parameters, but it is widely accepted that performance is tightly coupled to microbial community structure (McMahon et al., 2001; Dearman et al., 2006; Lee et al., 2010). However, changes in community structure were shown also to occur without detectable changes in performance (Fernandez et al., 2000; Dearman et al., 2006). Thus, the link between community structure and performance is unclear and more studies are needed (Bouallagui et al., 2005). In addition only a few studies investigating anaerobic microbial community structure have focused on simultaneously seeding replicate reactors (Fernandez et al., 1999; Fernandez et al., 2000; Hasham et al., 2000), leaving thus little space for factorial experimentation. Despite these limitations, microbial diversity *per se* was shown to be of lesser importance for the biogas production than was microbial community structure and its community flexibility (Dearman et al., 2006; Lee et al., 2010).

Batch systems such as biochemical methane potential (BMP) or anaerobic toxicity assays (ATA) (Owen et al., 1979) have been routinely used as microbial model systems to determine optimal reaction conditions or compound toxicity due to the level of experimental control they offer in factorial experiments (Jessup et al., 2004; Drake et al., 1996; Owen et al., 1979). Nyberg et al. (2008) and Fernandez-Cegri et al., 2010) have used molecular profiling in attempt to link microbial communities and functional characteristics in ATA and BMP. In their studies, however, the structure of microbial communities present in negative and positive controls (Fernandez-Cegri et al., 2010; Nyberg et al., 2008) and the starting community structures (Nyberg et al., 2008) have not been determined. To fill this gap, we analyzed and compared methanogenic community shifts in association with process data under simulating batch start-up conditions using three different biomass inocula. Further, the relationship between the general microbial community flexibility in

BMP and initial biogas production rates was established as a novel parameter to diagnose anaerobic processes, particularly under dynamic conditions like start-up.

2 MATERIALS AND METHODS

2.1 Inoculum and substrate collection

Biomasses were obtained from three full-scale digesters receiving different substrates: A - sewage sludge, B - urban organic waste and C - agricultural organic waste. Inoculum samples were collected aseptically in 20 L containers and immediately transferred to laboratory. None of these biomasses had been previously exposed to brewery-spent grain.

The brewery-spent grain was obtained from the brewery plant. Substrate was collected in 20 L plastic containers and stored at 4°C until use (< 5 days). Total organic carbon (TOC) (106.8 g / kg) and Total Kjeldahl nitrogen (TKN) (13.511 g / kg) were determined according to the APHA methods (APHA, 2005) and SIS EN 25663 (1996), respectively. The C:N ratio was 7.9 and the NH_4^+ content 0.21 g / kg. Brewery spent grain contains cellulose, hemicelloulose and also lignin, which are known for slow degradability under anaerobic conditions. Composition of these substances in brewery-spent grain varies and ranges from 16.8–25.4% for cellulose, 21.8–28.4% for hemicellulose (mostly arabinoxylans) and 11.9–27.8% for lignin (Mussatto et al., 2006). Lignin derivatives with aldehyde groups or apolar substituents are highly toxic to methanogens and because of this biodegradation of lignocellulosic substrates is quite difficult (Chen et al., 2008).

2.2 Reactors setup

The experiment was conducted essentially according to SIST EN ISO 11734:1999 standard and independently replicated twice. Oxitop bottles (1000 mL) equipped with pressure sensors (WTW, Germany) were used as anaerobic batch reactors. Side neck sampling ports were sealed with butyl rubber stoppers to prevent gas leakage.

The inoculum biomass concentration was adjusted to 2g of volatile solids per liter (VS/L). Inoculum with no carbon substrate addition served as negative control of the basal metabolic activity. Positive control and experimental inoculums were supplied with 50 mg TOC of glucose or brewery spent grain per g VS inoculums (TOC / g VS inoculum). The Oxitop bottles were flushed with nitrogen gas for 15 minutes to ensure anaerobic. The bottles were rotated at 120 rpm during incubation at 37°C (Infors, Switzerland). Hourly measurements of total biogas produced during the 14 days of incubation were recovered from pressure-sensor data loggers. The headspace gas composition was measured on days 0 and 14 by gas chromatograph (Shimadzu, GC14A-TCD). The gases were separated using 4 m long steel column with inner diameter of ¼ inches, filled with Porapak Q. Helium was used as a carrier gas. Injector and column temperature was 30° C and the detector temperature was 80° C. Calibration gas mixtures contained hydrogen, nitrogen, methane and carbon dioxide. Chromatographic signals were evaluated by integrator Chromatopack CR-4A (Shimadzu) based on an absolute calibration. pH was measured on days 0 and day 14 using pH meter (Orion 520A). Initial biogas production rates were calculated from biogas accumulation curves through a curve fitting approach using linear regression in STATISTICA & (StatSoft Inc, USA).

2.3 Microbial community structure

Genomic DNA was extracted using UltraClean Soil DNA Isolation kit (MOBIO, USA) according to the manufacturer instructions for maximum DNA yield. The quality of DNA was checked by 1 % agarose gel electrophoresis. PCR amplification was performed using MyCycler (BIO-RAD laboratories, USA). The PCR primers 6-FAM labeled 109f (6-FAM, 5’ - CAN GCT CAG TAA CRC GYR-3’) and 691r (5’-CGA TTA CAR GAT TTC AC-3’) were used to amplify the 16S rRNA gene of methanogenic archaeal community (Watanabe et al., 2004) Three replicate PCR reactions were performed for each DNA extract and subsequently pooled essentially as described before (Stres et al., 2008) After the initial denaturation (5 min 94°C), a total of 35 cycles, each including 60 s at 94°C, 60 s at 53°C, and 60 s at 72°C, was followed by a final extension step of 10 min at 72°C and PCR products were examined by electrophoresis on 1.5 % agarose gels. Residual primers were removed

using High Pure PCR Product Purification Kit (Roche, Switzerland) according to manufacturer instructions.

To provide a rationale in choosing gene-primers-enzyme combinations with highest resolution in T-RFLP analysis all restriction enzymes (http://rebase.neb.com/rebase/rebase.html) were tested on 120.000 archaeal sequences (Ribosomal Database Project II, http://rdp.cme.msu.edu/, Release10.05) using BESTRF software as described before (Stres et al., 2009).

T-RFLP analysis was conducted essentially as described before (Stres et al., 2009). Digestion of 50 ng of PCR products was carried out in a 30 μL volume using 3 μL of red buffer, 0.5 μL of HhaI (10 U μL−1, Fermentas Inc, USA) in separate reactions. The volume was adjusted to 30 μL with nuclease-free water (Sigma-Aldrich) and incubated at 37°C overnight. Digestions were inactivated for 15 min at 85 °C and purified by ethanol precipitation (Stres et al., 2008, Likar et al., 2009). Separation, detection and basic GeneScan analysis of fluorescently labeled T-RFs were performed on an automated ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems Inc.). Purified digested products were mixed with 0.5 μL internal-lane DNA standard (Genescan 500 ROX, Applied Biosystems Inc.) and 10 μL deionized formamide. Before analysis, DNA samples were denatured for 2 min at 95 °C and immediately placed on ice. T-RFLP patterns with total fluorescence exceeding the limit of 10.000 units were analyzed and peaks representing 0.5% or less of the total community signal were excluded from further analyses by BioNumerics software (AppliedMaths, Sint-Martens-Latem, Belgium). Pearson's correlation coefficient with UPGMA clustering (for unweighted pair-group method with arithmetic averages) was used to derive general similarity of community profiles and the significance of clusters was determined using cluster-cutoff tool in BioNumerics (Stres et al., 2008, Likar et al., 2009).

Variation partitioning was conducted in CANOCO 4.5 to extract the most important parameters explaining variation in microbial community data (Stres et al., 2010). Detrended correspondence analysis was performed to determine whether unimodal or linear models fitted the community datasets better. Redundancy analysis (RDA) with forward selection was then used first to determine experimental parameters

significantly associated with community data to eliminate co-linear variables. The significance of the final RDA was determined by 999 Monte Carlo permutations. Data collected in BMP experiment served as explanatory variables. The association between microbial community flexibility (defined as difference in Pearson's similarity between initial and developed microbial community fingerprints) and various BMP parameters was tested by multiple linear regression in STATISTICA 6 (StatSoft Inc.).

3 RESULTS AND DISCUSSION

Initial methanogenic community in inoculum A differed significantly (54% dissimilarity) from inoculums B and C that shared a much larger fraction of communities (only 17% dissimilarity) (Fig. 17) and formed one separate cluster from inoculum A community supported by cluster cut-off values. This general distinction between inoculum A and the BC cluster was evident also at the end of BMP assay, as developed communities remained separated in two clusters (Fig. 17). This suggests that significant starting differences in microbial communities were also recovered after incubation in BMP assay.

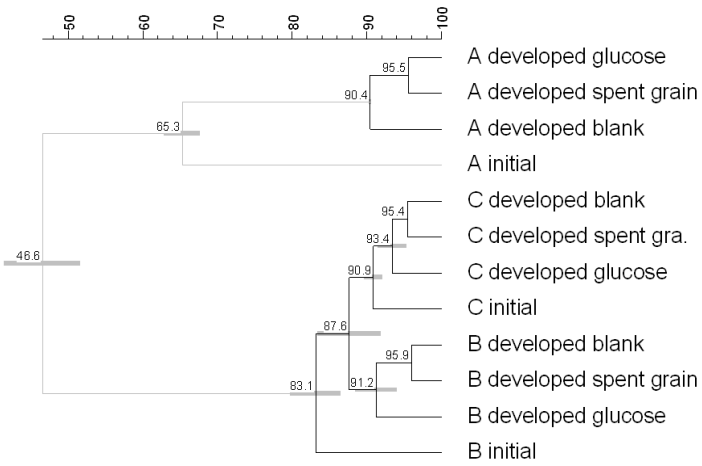

Fig. 17**:** *Pearson correlation dendrogram of T-RFLP fingerprints of methanogenic microbial communities originating from digesters treating sewage sludge (A), municipal organic waste (B) and agricultural waste (C). Initial – inoculum at the onset of BMP assays; blank, glucose, spent grain– developed microbial communities without substrate or supplied with glucose or 100 mg spent grain TOC / g* $VS_{inoculum}$*. Horizontal gray lines delineate relevant clusters separated by the cluster-cut off method and similarity values (cophenetic correlation) are shown as numbers at each branch. Horizontal bars (error flags) represent the reliability and internal consistency of the branch.*

Developed inoculum B and C were much more similar and branched closely to respective initial inoculum communities (17% and 10% dissimilarity, respectively) (Fig. 17). Developed microbial communities of inoculum A differed from initial community (35% dissimilarity), formed a separate cluster supported by cluster cut-off values and thus showed high response to novel conditions in BMP assay. It is important to note that microbial communities of either inoculum degrading novel substrate (spent grain) developed highly similar communities to respective glucose or no substrate BMP assay controls (>91% similarity). Further, RDA identified incubation in BMP assay and inoculum as the key experimental parameters explaining 82.5% of the total variance in microbial community data ($P < 0.02$). These results show that for the correct distinction of effects of novel substrates in BMP assay or toxic compounds in ATA (Owen et al., 1979) from the general incubation effects observed in positive and negative controls, factorial design of experiments is needed. In published literature, the start-up of unreplicated batch mode operated lab-scale reactors (Lee et al., 2010, Fernandez et al., 2000) is described (Annex 1).

To our knowledge, this may be one of the first studies, which provided methanogen community dynamics in all routine BMP assay variants for three distinct inoculums and statistically compared the community shift profiles. It also extends well to ATA, as reanalysis of published data reversed the outcome of previous studies (Nyberg et al., 2008) and showed that the effects of different substances on microbial communities could be detected (Annex 2).

Further, archaeal community flexibility was shown to correlate with stable biogas production in lab-scale operated reactors (Fernandez et al., 1999; Fernandez et al., 2000; Hasham et al., 2000), and during batch-scale start-up of lab-scale reactors (Dearman et al., 2008, Lee et al., 2010). However, the relationship between community flexibility and measured parameters in batch-scale BMP assay has not been determined. In our study, biogas production rate from novel substrate spent grain (Table 2) and general community flexibility observed in BMP (Fig. 17) showed a significant linear relationship ($P > 0.94$, $R2 = 0.98$) (Fig. 18).

Table 2**:** *Results of spent grain degradation in biochemical methane potential assay using microbial communities originating from digesters treating sewage sludge (A), municipal organic waste (B) and agricultural waste (C).*

Parameter	A	B	C
initial biogas production rate (mL / d)	22.1 ± 2.5	7 ± 1.3	3 ± 2.1
methane yield (mL CH4 / g TOC spent grain)	193 ± 15.5	215 ± 15.5	130 ± 12
spent grain degradation (%)	55 ± 9	65 ± 4	40 ± 6
pH	7.2 ± 0.1	7.1 ± 0.1	7.4 ± 0.3

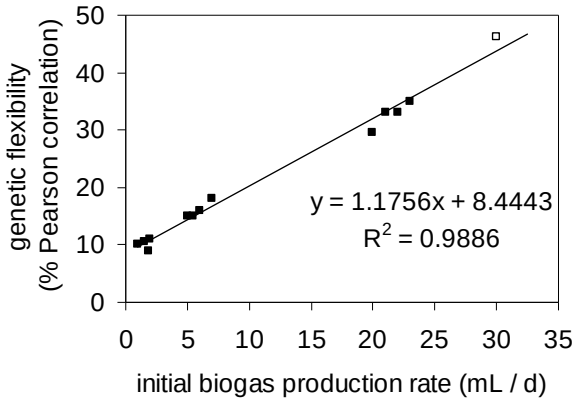

Fig. 18**:** *The relationship between initial biogas production rate and overall genetic flexibility observed in biochemical methane potential assays in experiments on three distinct inoculums. □ - data recalculated from Lee et al. (2010).*

Other physiological parameters measured (methane yield coefficient, grain degradation efficiency, pH) were not significantly related to general community flexibility in BMP. Thus the relationship between the general community flexibility in BMP assay and initial biogas production rates can be used as a novel parameter to diagnose anaerobic processes, particularly under dynamic conditions like start-up.

3.1 Conclusions

A general positive relationship between archaeal community flexibility and its initial biogas production rate was identified as a novel predictor of inoculum suitability in digester start up. The coupling of methane potential test and T-RFLP analysis of methanogenic microbial community on the level of archaeal 16S rRNA genes suggests these two approaches could be applied in biogas production management routinely as additional measure of anaerobic microbial biomass suitability for the degradation of specific substrates or substrate mixtures.

CHAPTER 3

CHAPTER 3 - CYANIDE DEGRADATION IN TWO CONTRASTING ANAEROBIC DIGESTER BIOMASS SAMPLES

1 INTRODUCTION

Cyanide is carbon nitrogen radical, which can be found in low concentrations in a wide range of various life forms including photosynthetic bacteria, algae, fungi, plants and foods despite its toxicity (Dash et al., 2008). Cyanide is commonly found as a contaminant in wastewaters from metal finishing industries, mining pharmaceuticals and also food industry. The industrial effluents generally contain between 0.01 and 10 mg/L of total cyanide, meanwhile in unpolluted rivers and lake waters cyanide concentrations are 100 – 500 times lower and range from 0.0001-0.05 mg/L (Wild et al., 1994). It is estimated that its production from this industries is approximately 14 million kg/yr (ATSDR, 1997).

Cyanide occurs in many different forms in water, and the specific form of cyanide determines its reactivity and toxicity. The form of cyanide also determines the management of cyanide contaminated organic waste. Chemically cyanide is classified into organic and inorganic forms. Inorganic forms occur in all three physical states (solid, aqueous and gas). The most common gaseous form of cyanide is hydrogen cyanide (HCN), which is present in wastewater discharges and industrial leachates and can be formed also in nature. HCN gas is colorless with an odor of bitter almonds. It is highly toxic to humans. It is very soluble in water, forming a weak acid, HCN (aq), upon dissolution. It also has high vapor pressure and is readily volatilized from water at pH values less than 9 (ATSDR, 1997). Aqueous forms of cyanide appear as free cyanide, metal-cyanide complexes, hydrogen cyanate HCNO, cyanate CNO^- and thiocyanate SCN^- species and organocyanide compounds (Ghosh et al., 1999).

Free cyanide is the most toxic form and it appears as soluble hydrogen cyanide (HCN) aq or soluble cyanide anion (CN^-) At pH values less than, 9 HCN is the dominant free cyanide species, while at higher pH values cyanide ion dominates free cyanide. The cyanide is a very good ligand and many metals can associate with the cyanide anion to form dissolved metal-cyanide complexes, which are generally less

toxic. Metal cyanide complexes are divided in two groups; weak metal cyanide complexes (Ag, Cd, Cu, Ni, Hg, Zn) and strong metal cyanide complexes (Fe, Co, Pt). Free cyanide can be oxidized to form cyanate, CNO^-, which is substantially less toxic than free cyanide. Furthermore, free cyanide can react with various forms of sulfur to form thiocyanate SCN^-, which is relatively nontoxic (seven times less toxic than free cyanide), but can form complexes with many metals.

Anaerobic microbial degradation of cyanide and cyanogenic compounds has been studied in the last two decades (Fallon et al., 1991, Fedorak and Hrudey 1989, Annachhatre et al., 2000, 2001 Gijzen et al., 2000, Paixao et al., 2000, Quan et al., 2004) with some promising results. There are four general pathways for the cyanide biodegradation: hydrolytic, oxidative, reductive, and substitution transfer (Ebbs et al., 2004). According to the literature under anaerobic conditions reductive or hydrolytic pathway are present (Fallon, 1992, Kunz et al., 1992). Metabolic pathways are not only influenced by the presence of cyanide, but also by pH, carbon source and oxygen partial pressure (Ebbs et al., 2004).

The reductive pathway is mediated by nitrogenase enzyme. The enzyme utilizes HCN and produces methane and ammonia as the end products (Gupta et al., 2010). However, as the enzyme (nitrogenase) required for such pathway is very rarely found in organisms, the fraction of cyanide converted using this pathway is believed to be very small.

There are five enzymes present in microbial systems that catalyze the hydrolytic pathway: (i) cyanide hydratase, (ii) nitrile hydratase, (iii) carbonyl pathways (thiocyanate hydrolase), (iv) nitrilase, and (v) cyanidase. First three enzymes have a specific substrate and directly hydrolyze and cleave the carbon–nitrogen triple bond to form formamide, and last two convert it to ammonia and formic acid, which are utilized in the metabolism activity (Gupta et al., 2010).

The degradation pathways are primarily utilized by prokaryotic organisms when their presence in contaminated media (e.g wastewaters form gold mining, metal plating etc…) provided a selection pressure that enriches cyanide degrading organisms in the microbial consortia or those tolerant or capable of its detoxification.

Based on the information in rare published literature on anaerobic degradation of wastewaters contaminated with cyanide, anaerobic degradation is possible following the proper adaptation period. However, the widespread observation of cyanide toxicity to methanogens has resulted in minimal utilization of this technology, in spite of the potential for reduced operating costs versus aerobic treatment (Dash et al., 2009).

The first modern report of anaerobic biological cyanide transformation was made by Fedorak and Hrudey (Fedorak and Hrudey 1989) for semi-continuous batch cultures with a 25-day hydraulic retention time. Semi-continuous anaerobic phenol-degrading cultures were exposed to a range of total cyanide concentrations from 5 to 30 mg/L for a 140-day period. The higher cyanide concentrations resulted in longer lag periods before the onset of methane production. Most methanogenesis occurred after the free cyanide had been significantly reduced. The major product of cyanide transformation could not be identified, although bicarbonate was found as a minor product.

Fallon (1992) demonstrated that cyanide was anaerobically hydrolyzed to form ammonia and formate, while the latter was subsequently converted to bicarbonate. The appearance of bicarbonate was correlated with higher rates of cyanide transformation. The anaerobic systems described in the study showed good, long-term removal of cyanide in presence of a variety of feed media. Systems retained metabolic activity at cyanide concentrations of up to about 300 mg/L, which appeared to be higher than reported previously for anaerobic microbes under controlled conditions.

Gijzen et al., (2000) demonstrated that anaerobic sludge must be adapted to the cyanide to enable its stable long term conversion. First addition of CN^- at 5 mg/L and subsequent sudden increases in influent CN^- concentrations during the acclimatization process resulted in temporary deterioration of reactor performance in terms of methane production and COD conversion, while CN^- concentrations in the effluent were temporarily increased. Recovery from CN^- inhibition was observed within 3-4 weeks, when effluent CN^- concentrations decreased again below about 10

mg/L. However, once the sludge was adapted to cyanide concentration further step-wise increases of CN^- concentration up to 125 mg/L were possible, while maintaining high methane production and COD degradation efficiency. This clearly demonstrated that sludge can be adapted to high cyanide concentrations, although the results of adaptation at the cellular level of individual microbe (bacteria or archaea) remained unclear. The results of sludge activity tests in the Gijzen study suggest that hydrogenotrophic methanogens were less sensitive to cyanide compared to acetoclastic methanogens. However, as researchers in these studies focused on the effects and degradation of cyanide, there had been no report on microbial community dynamics during the process of cyanide removal. We set to fill this gap with our research work.

Quan et al. (2004) were the first to describe the characteristics of microbial community of a cyanide-degrading consortium that efficiently removed nickel-complexed cyanide under sulfate-reducing conditions. Consortium completely removed 0.5 mM of nickel-complexed cyanide under sulfate-reducing conditions in 11 days. Analysis of clone library of 16S rRNA genes showed that the consortium was composed of three major phylotypes identified as NC1, NC2, and NC3. The group NC1 was a member of sulfate reducing bacteria of *Desulfovibrio*, resembling *Desulfovibrio magneticus* with 99.4% similarity. NC2 was closely related to an uncultured bacterium 2C (Richardson et al. 2002) (97.7%), which was from anaerobic trichloroethene (TCE) dechlorinating enrichment. NC3 was close to clone AA56 (96.3% similarity), which became dominant in an anaerobic digester degrading lactate (Delbès et al. 2000).

However, complexed cyanides are known to be generally less toxic for microorganisms than free cyanide (CN^-) (Gijzen et al., 2000, Fallon et al., 1991).

Thus, our research was focused on free cyanide (CN^-) degradation under methanogenic conditions at pH 7 as to the best of our knowledge there has been no published analysis describing fully the microbial consortia able to degrade CN^- in different concentrations under methanogenic conditions. We focused our research on structural and functional aspects of the key microbial communities using physiological and molecular approaches to bridge this gap.

2 MATERIALS AND METHODS

2.1 Experiment overview

During research work with cyanides we conducted five experiments. We used two different microbial biomasses from full-scale biogas reactors (two experiments with biomass from CSTR and two experiments with biomass from UASB reactor) as shown in Table 3. Materials and methods for all four experiments are described below.

***Table 3*:** An overview of experiments

	CN concentration	WWTP	Industrial	Page #
Experiment 1	1.5 mg /L CN^-	CSTR		52
Experiment 2	5 and 10 mg /L CN^-	CSTR		55
Experiment 3	adaptation experiment: 2 mg /L applied 3 times *(1.5, 2 and 3.5 mg/L sub experiment)	CSTR		66
Experiment 4	1, 2, 3 mg /L CN^-		UASB	75
Experiment 5	5 and 8.5 mg /L CN^- (sterilization and Kiliani Fischer sub experiment)		UASB	81

2.2 Inoculum and substrate collection

For the 1st, 2nd and 3rd experiment microbial biomass from continuous stirred tank reactor (CSTR) biogas reactor was used as inoculum. The mother biogas reactor has been operated for more than 25 years at 38°C and loaded with municipal sludge from aerobic wastewater treatment at a loading rate 2.1g COD/L/day and hydraulic retention time of 48 days. The soluble organic carbon removal efficiency measured by COD was greater than 65 %. Inoculum was collected in 10 L plastic containers and thermostated at 38°C for 5 days to remove any residual carbon source (e.g. carbon source for microorganisms). TS (24.21 g / L), VS (50.3 %) and COD of biomass were determined according to the APHA standard methods (APHA, 2005).
For 4th and 5th experiment we used anaerobic granulated biomass obtained from industrial UASB (Up flow Anaerobic Sludge Blanket) reactor. The UASB reactor has been operated for more than 2 years at 37°C treating brewery wastewaters (BWW) at a loading rate 4g COD/L/day and hydraulic retention time of 40 hours.

The soluble organic carbon removal efficiency measured by COD was higher than 90 %. TS (43.7 g / L), VS (48.4 % of TS) and COD of microbial biomass were determined according to the APHA standard methods (APHA, 2005).

2.3 Reactors setup and analytical methods

All of the experiments were conducted essentially according to SIST EN ISO 11734:1999 standard and independently replicated twice. Oxitop bottles (1000 mL) equipped with pressure sensors (WTW, Germany) were used as anaerobic batch reactors. Side neck sampling ports were sealed with butyl rubber stoppers to prevent gas leakage.

For the 1st experiment reactors were inoculated with 100 mL of microbial biomass from CSTR reactor. All of the samples except blank, which served to monitor the basal metabolic activity, were amended with 2 mL of 0.83 M glucose (carbon source), 20 mL of phosphate buffer and diluted freshly deoxygenated water was added to the 500 mL mark. The initial cyanide concentration in reactor volume was 1.5 mg/L of KCN. KCN was used due to a high solubility in aqueous solutions and releasing of free cyanide anion (CN^-). Hourly measurements of total biogas produced during 21 days of incubation were recovered from pressure-sensor data loggers and served as descriptors of the initial lag phases, the biogas production rate and the total amount of biogas produced. The headspace gas composition was measured on days 6, 13 and 21 by GC-MS as described below (2.4).

2nd experiment was conducted in the same way as 1st, except that initial cyanide concentration in reactor volume was increased to 5.0 and 10mg/L in and was incubated for 29 days. The headspace gas composition was measured on days 1, 8, 15, 21 and 29 by GC-MS

3rd experiment was conducted in the same way as 1st, except that initial cyanide concentration in reactor volume was 2.0 mg/L and was incubated for 63 days with two subsequent applications of 2.0 mg/L of KCN every 21 days. The purpose of three consecutive cyanide amendments was a stepwise adaptation of microbial community to higher cyanide concentrations that we used in 2nd experiment. The headspace gas composition was measured on days 6, 13, 21, 25, 28, 37, 42, 46, 52 and 63 by GC-MS

For the 4th experiment reactors were inoculated with 40 mL of biomass from UASB reactor. All of the samples except blank, which served to monitor the basal metabolic activity, were amended with 60 mL of brewery waste water (180 mg of COD as carbon source), 20 mL of phosphate buffer and diluted freshly deoxygenated water was added to the 500 mL mark. The initial cyanide concentration in reactor volume was 1.0, 2.0 and 3.0 mg/L of KCN. Hourly measurements of total biogas produced during 21 days of incubation were recovered from pressure-sensor data loggers and served as descriptors of the initial lag phases, the biogas production velocity and the total amount of biogas produced. The headspace gas composition was measured on days 5, 14 and 21 by GC-MS

5th experiment was conducted in the same way as 3rd, except that initial cyanide concentration in reactor volume was 5.0 and 8.5 mg/L of KCN and it was incubated for 60 days. The headspace gas composition was measured on days 7, 14, 21, 28, 35 and 60 by GC-MS. Sterilization sub experiment was performed in order to determine the proportion of biotic and abiotic conversion of cyanide.

2.4 GC/MS analyses

The headspace gas composition for all five experiments was measured by gas chromatograph (AGILENT 3000 A Micro GC-TCD) in combination with quadrupole mass spectrometer (PFEIFFER PRISMA PLUS QME 220). GC was equipped with three parallel columns. The carrier gas was argon with a flow of 100 mL / min or 6 L / min. Injection temperature was 130˚C, working pressure was 1 bar, injector retention was 90 ms. Temperatures and pressures of the individual columns differed:

PLOT U: 100 ˚C; 2,068 bars,
(silicon capillary column with combined stationary phase divinylbenzene and ethylene glycol dimethacrylate)

PLOT Q: 70 ˚C; 2,068 bars,
(robust column with a stationary phase consisting of polystyrene and divinylbenzene)

PLOT MS: 80 ˚C; 2,206 bars
(molecular sieve)

Table 4: *Retention times of analyzed gases (t)*

Column	t/min	Gas
PLOT U	1.424	H_2O
	0.767	H_2S
	2.689	HCN
PLOT Q	1.214	H_2O
	1.144	H_2S
	1.971	HCN
	0.727	CO_2
PLOT Molecular Sieve	0.739	H_2
	1.775	CH_4
	1.195	N_2

Inlet pressure in the QMS was 1 mbar, the working pressure was 1.00 × 10-6 mbar. Integration of individual peaks was made by OriginPro 8th edition (Originlab, USA). VQMS calibration data were obtained using the six-way valve on the calibration loop at the injection of the test gas in the GC/QMS. In all cases 1 mL of gas was injected eleven times and the response amplitudes were averaged. For GC calibration the following gas mixtures were used:

- Ar–CO_2 (96-4 vol.%),
- Ar–CO_2–CH_4 (92-4-4 vol.%),
- Ar–CH_4 (96-5 vol.%),
- Ar–CH_4 (96-4 vol.%),
- Ar–H_2S (1000 ppm),
- Ar–CH_4–CO–CO_2–H_2 (84-4-4-4-4 vol.%).

Data obtained from GC/QMS were processed using Microsoft Excel and Origin 8.0. Calibration data of gases is given in (Table 5).

Table 5: *Calibration data of gases*

	I_{QMS}/A.min.mol^{-1}	σ/A.min.mol^{-1}
H_2	$1{,}958 \times 10^{-6}$	$\pm 3{,}92 \times 10^{-8}$
CO_2	$2{,}049 \times 10^{-7}$	$\pm 4{,}10 \times 10^{-9}$
CH_4	$3{,}130 \times 10^{-7}$	$\pm 6{,}26 \times 10^{-9}$
CO	$3{,}038 \times 10^{-7}$	$\pm 6{,}08 \times 10^{-9}$

2.5 Confirmation of gaseous HCN detection on GC/MS

For the determination of the retention time of gaseous HCN, a water solution of sodium cyanide was prepared in the pressure bottles (OxiTop) at different pH values. Namely, the boiling point of hydrogen cyanide at a pressure of 101.3 kPa is 26 °C. Since pK_a of HCN is 9.24 (Sehmel et al., 1989), the equilibrium between gaseous HCN and CN^- in the solution is strongly pH dependent. A volume of 450 mL of distilled water was added to the OxiTop bottle, purged with argon and 2.40 mL of 0.20 M KCN was added trough septum. The pH value of the resulting solution was 8.5. The GC apparatus did not detect any HCN gas. In the next experiment the pH value of the sodium cyanide solution was lowered by the addition of a known predetermined volume of 0.10 M HCl to 5.0. The GC analyzer detected HCN on Plot U at a retention time of 2.69 min and on Plot Q at a retention time of 1.97 min.

2.6 Analysis of NH4-N

Ammonia concentrations in all experiments were measured by LCK 305, 302 Ammonium cuvette test (Hach Lange, Germany) using Hach-Lange DR2800 VIS spectrophotometer according to the manufacturer instructions. Ammonium ions react at pH 12.6 with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol blue, which is determined photometrically at 560 nm. Spectrophotometer was calibrated according to the ISO

8466-1 and DIN 38402 A51 standards „Calibration of analysis methods“. The detection and the quantitation limits were determined in conformity with DIN 32645.

Table 6: *Technical data for cuvette test LCK302 (Results as NH4-N)*

Detection limit	0.8 mg/L
Quantitation limit	2.4 mg/L
Sensitivity	0.049 Abs./(mg/L)
Ordinate intersect	0.128 Abs.
Residual standard deviation	0.0073 Abs.
Method variation coefficient	1.49 %
Method standard deviation	1.5 mg/L
Confidence intervall (95%)	± 3.7 mg/L

Table 7: *Technical data for cuvette test LCK305 (Results as NH4-N)*

Detection limit	0.033 mg/L
Quantitation limit	0.099 mg/L
Sensitivity	0.140 Abs./(mg/L)
Ordinate intersect	0.092 Abs.
Residual standard deviation	0.0019 Abs.
Method variation coefficient	1.63 %
Method standard deviation	0.13 mg/L
Confidence intervall (95%)	± 0.33 mg/L

2.7 Analysis of cyanide

Free cyanide concentrations in all experiments in Chapter 3 were measured by LCK 315 cyanide cuvette test Pyridine-Pyrazalone Method (Epstein et al., 1947) (Hach-Lange, Germany) according to the manufacturer instructions. The principle of determination is that cyanides react with chlorine to form cyanogen chloride, which in turn reacts with pyridine in the presence of barbituric acid, condensing to form a violet colorant, which is determined photometrically using Hach-Lange DR2800 VIS spectrophotometer at 590 nm. Spectrophotometer was calibrated according to the ISO 8466-1 and DIN 38402 A51 standards „Calibration of analysis methods“. The detection and the quantitation limits were determined in conformity with DIN 32645.

Table 8: *Technical data for cuvette test LCK315*

Detection limit	0.002 mg/L
Quantitation limit	0.007 mg/L
Sensitivity	2.725 Abs./(mg/L)
Ordinate intersect	0.059 Abs.
Residual standard deviation	0.0074 Abs.
Method variation coefficient	0.82 %
Method standard deviation	0.003 mg/L
Confidence intervall (95%)	± 0.007 mg/L

Total cyanide was analyzed according to the APHA standard methods (APHA, 2005).

2.8 Analysis of COD

COD in all experiments in Chapter 3 were measured by LCK 514 COD cuvette test (Hach-Lange, Germany) according to the manufacturer instructions. The principle of determination is that oxidizable substances react with sulphuric acid – potassium dichromate solution in the presence of silver sulphate as a catalyst. Chloride is masked by mercury sulphate. The green coloration of Cr^{3+} is evaluated photometrically using Hach-Lange DR2800 VIS spectrophotometer at 605 nm. Spectrophotometer was calibrated according to the ISO 8466-1 and DIN 38402 A51 standards „Calibration of analysis methods“. The detection and the quantitation limits were determined in conformity with DIN 32645.

Table 9: *Technical data for cuvette test LCK514*

Detection limit	4.6 mg/L
Quantitation limit	13.7 mg/L
Sensitivity	0.0005 Abs./(mg/L)
Ordinate intersect	0.063 Abs.
Residual standard deviation	0.0017 Abs.
Method variation coefficient	0.33 %
Method standard deviation	3.5 mg/L
Confidence intervall (95%)	± 8.7 mg/L

2.9 pH measurements

pH was measured in 20 mL samples without centrifugation immediately after sampling using pH meter (Orion 520A). pH meter was calibrated with two pH calibration buffers of exactly known pH.

2.10 Analysis of volatile fatty acids

Volatile fatty acids (VFAs C1-C5 molecules) were extracted from the samples with diethyl ether according to the procedure of Holdeman et al. (1977) and analysed by gas chromatograph Schimadzu GC-14A with split/splitless injector and flame ionization detector (FID), equipped with fused silica capillary column DB-WAX of 30 m length, 0.326 mm diameter and 0.25 μm stationary phase thickness (J&W Scientific, 123-7032). Quantification was performed by an internal standard method (addition of crotonic acid an VFAs to the sample). For calibration, we used a mixture of short chain fatty acids in the composition: acetic acid (0.525 g / L), propionic acid (0.495 g / L), iso-butyric acid (0.475 g / L), n-butyric acid (0.480 g / L), iso-valeric acid (0.465 g / L), n-valeric acid (0.470 g / L), n-capronic acid (0.465 g / L). The injector temperature was 160 °C, the detector temperature 210 °C, the column temperature 75 °C.

2.10 Analysis of metals in microbial biomass

Triplicate samples (10 mL) were wet digested in a closed PFA vial (561B, Savillex, Minnesota, USA) with 3 mL of HNO_3 (TraceSelect Fluka, Buchs) and 2 mL of H_2O_2 (Suprapur Merck, Darmstadt). Complete digestion of the samples was performed at 200 °C for 1 h. After 1 h vials were opened and samples were heated until almost dry. Digested samples were diluted to 10 mL with Milli-Q water and analyzed with Agilent 7500ce series ICP-MS instrument. Standard addition technique (N = 10) was used to avoid matrix interferences. Limit of detection was calculated as concentration corresponding to three-fold standard deviation (3s, N = 6) of blank determinations. Blanks were subjected to the same digestion procedure as samples.

3 RESULTS AND DISCUSSION

3.1 Experiment I: CSTR sludge amended with 1.5 mg/L of KCN

Aim: The aim of the first experiment was to observe response of selected biomass to CN^- application. We started with initial concentration 1.5 mg/L of KCN in first experiment to see if the chosen system operates with newly selected biomass from full scale CSTR biogas reactor. Low concentration of KCN was selected (1.5 mg/L) since there was no report about inhibition of the anaerobic process at concentrations below 2 mg/L of free cyanide. Fedorak and Hrudey (1989) reported about inhibition of the process at concentration 2.0 mg/L. Short lag phase of approximately 60 hours was observed in the case of cyanide amended sample.

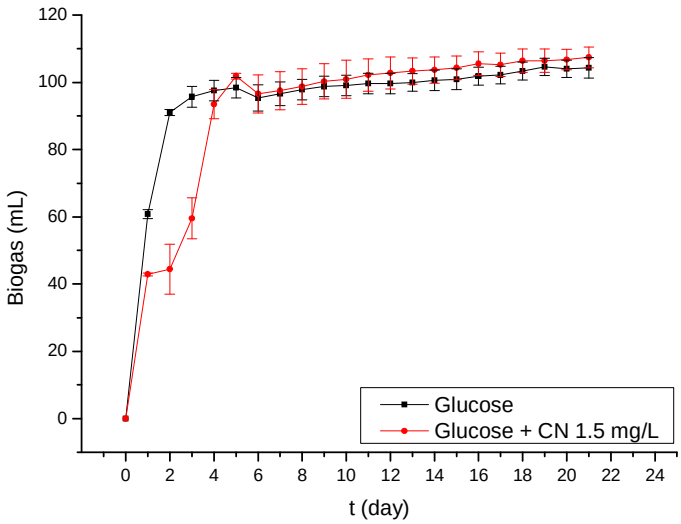

Fig. 19**:** *Cumulative biogas production. Values are means of three replicates ± SD.*

Following the lag phase (which lasted approximately 48 hours) normal methanogenesis compared to the positive control was observed as shown in Fig. 19. Methane content in cumulated biogas was 59.6 ± 0.5 % for cyanide sample and 60.27 ± 2.1 % for glucose - positive control sample (Fig. 20).

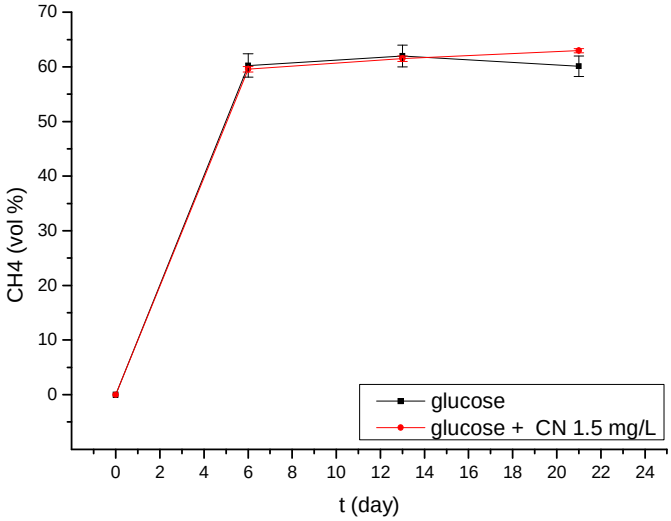

Fig. 20**:** *Methane content during 21 days of incubation. Values are means of three replicates ± SD.*

Free cyanide concentration decreased rapidly. After 6 days of incubation concentration of free cyanide was only 0.6 ± 0.03 mg/L. At the end of incubation period free cyanide concentration was 0.034 ± 0.02 mg/L (Fig. 21).

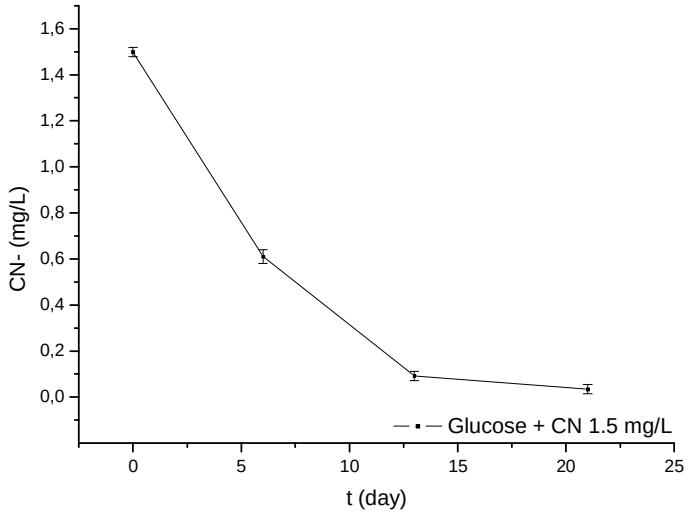

Fig. 21**:** *Concentrations of free cyanide during 21 days of incubation. Values are means of three replicates ± SD.*

Significant findings of the 1st experiment

First application of cyanide to selected microbial biomass resulted in unaffected methanogenesis based on methane content, cumulative biogas production and free cyanide removal. Such results were expected since there were no previous reports on inhibition at KCN concentration (1.5 mg /L) used in experiment.

3.2 Experiment II: CSTR sludge amended with 5 and 10 mg / L of KCN

Aim: The purpose of the second experiment was to observe response of selected biomass to much higher concentrations of KCN. We applied two different concentrations of KCN (5.0 and 10.0 mg/L of KCN) to test the response of selected biomass to concentrations that were reported to be inhibiting concentrations (Fedorak and Hrudey 1989, Gijzen et al., 2000).

Twenty lab-batch reactors (Oxitop) were tested during the period of 29 days to evaluate mesophilic anaerobic digestion of the cyanide (KCN) in two different

concentrations. KCN was added at the beginning of the experiment in concentrations 5 mg/L and 10 mg/L, respectively. Performance of the reactors is shown in Fig. 22. In the case of positive control (glucose) process completed successfully and high cumulative biogas production was recorded (149.4 mL). Contrary in the case of KCN addition, strong inhibition of process was observed as was reported before (Fedorak and Hrudey 1989, Gijzen et al., 2000). Both authors reported significant inhibition effect on methanogenesis at the application of CN^- in concentrations higher than 5 mg/L.

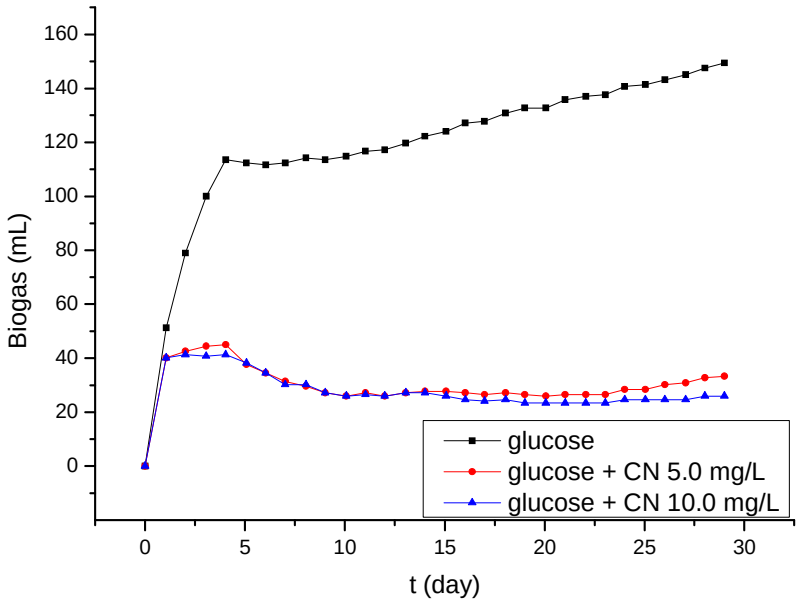

Fig. 22**:** *Cumulative biogas production during 29 days of incubation. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Cumulative biogas production in the experiments where cyanide was added to the substrates was 25.3 ± 1.4 mL and 25.9 ± 1.1 mL respectively, which is approximately 83.1 and 82.7 % less than in the case of the positive control with added glucose. This indicates a partial inhibition of methanogenesis, but not a complete shutdown as was reported by Fedorak and Hrudey (1989). In their batch anaerobic study with 2-2.5 mg/L of CN^- concentration in the medium, methane generation had started only after the complete depletion of CN^-. Chakraborty and Veeramani (2006) also reported total inhibition of methanogenesis at CN^- concentration 2.1 mg/L. In our case methane content did not exceed 40 (vol.%) at a

cyanide concentration of 5 mg/L and below 25 (vol.%) at a cyanide concentration of 10 mg/L as shown in Fig. 23. According to the results of methane content measurements in our case methanogenesis was definitely significantly inhibited but did not completely fail. These differences can be probably explained by differences in microbial biomass as was reported by Gijzen et al. (2000). Thicker biofilms are supposed to be capable of handling higher CN^- concentrations due to a minimal exposure of inner biofilm layer. Forming of biofilm can also explain more effective CN^- degradation in batch experiments (Gijzen et al., 2000, Fedorak and Hurdey 1989) in comparison with continuous systems in which biofilm is hardly formed because of the continuous flow.

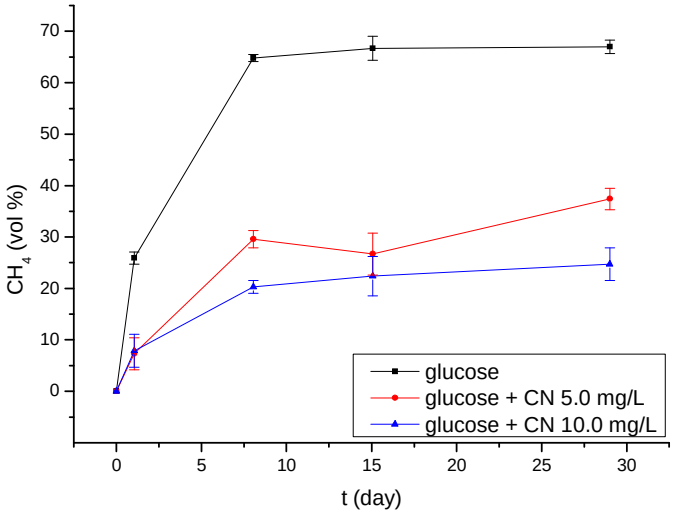

***Fig. 23**: Methane content during 29 days of incubation. Values are means of three replicates ± SD.*

Reactors performance in terms of removing COD (Fig. 24) was 92 % in case of glucose. Meanwhile in the presence of CN^- amended samples no COD removal could be detected. On the contrary, COD was apparently increasing until the end of experiment. This can be explained by CN^- toxicity to microorganisms, which caused accumulation of organic compounds derived from microbial biomass and consequent increase in COD. This is yet another indication of process inhibition and CN^- toxicity to the microbial community.

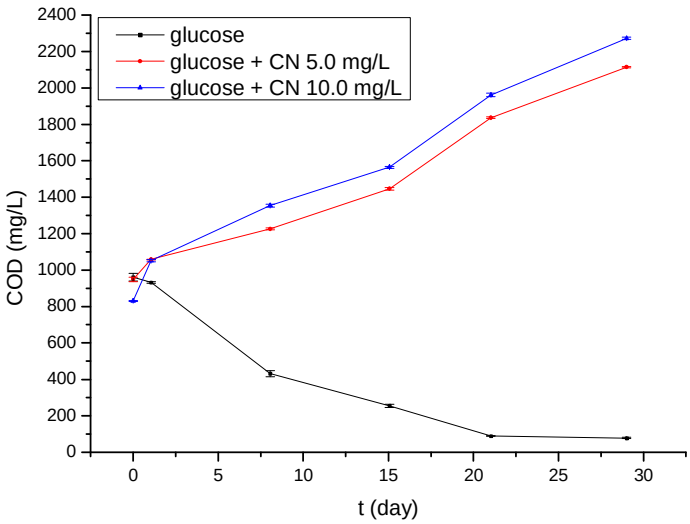

***Fig. 24**: COD concentrations in batch reactors during 29 days of incubation Values are means of three replicates ± SD.*

In the acidogenic phase monomers are degraded to short chain organic acids, (C1-C5 molecules; …acetic acid, propionic acid, butyric acid…). In all samples, the acetic acid represented the largest fraction as shown in Fig. 25. Other acids were also present (not shown), but in minor quantities.

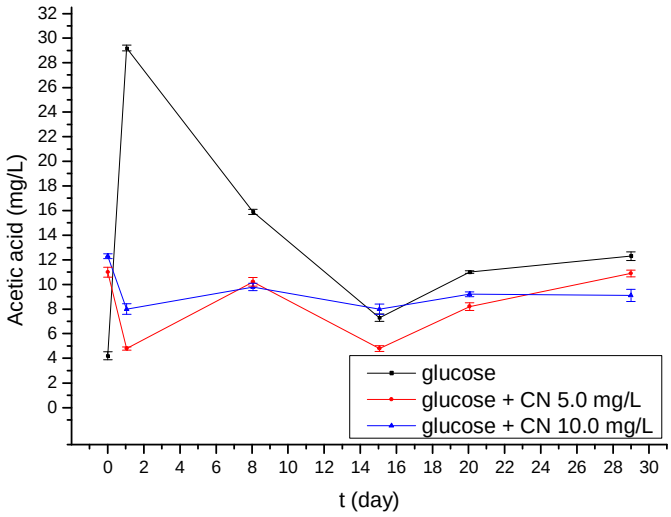

***Fig. 25**: Acetic acid concentrations in samples during 29 days of incubation. Values are means of three replicates ± SD*

In the case of glucose sample, we observed an increase in the concentration of acetic acid during the first days of incubation followed by a decrease. Acetic acid concentration was correlated to methane content as shown in Fig. 26. Meanwhile, in the case of cyanide samples, there was no accumulation of acetic acid. The concentration of the acetic acid in cyanide samples was the same during the whole period of incubation with minor fluctuations. This also correlates with methane production in both cyanide samples (for cyanide sample 5.0 mg/L, not shown) as shown in Fig. 27.

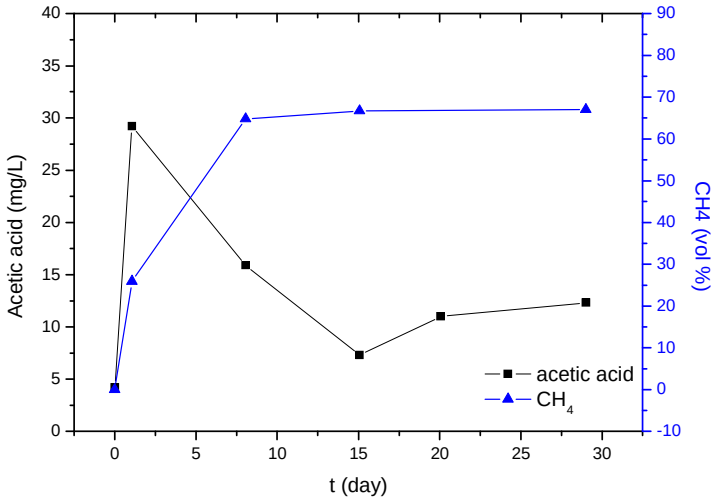

Fig. 26**:** *Correlation between acetic acid concentration and methane content in glucose sample. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

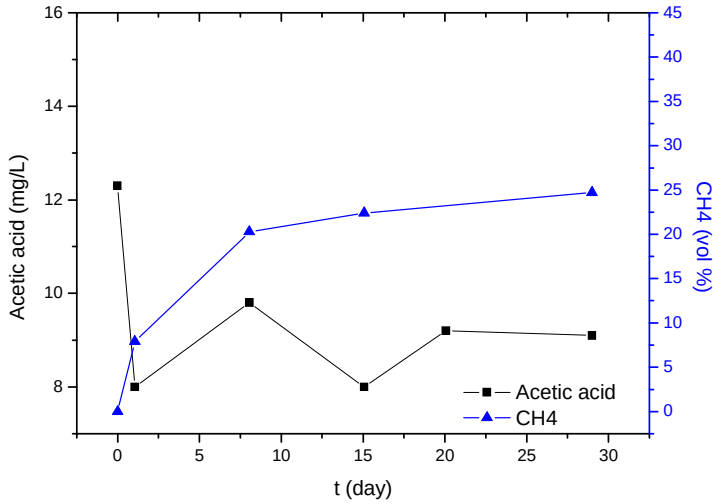

Fig. 27: *Correlation between acid concentration and methane content in cyanide sample with initial CN^- concentration 10 mg/L. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Concentrations of volatile fatty acids were too small to cause inhibition of the process. Acetic acid starts inhibition at 1000 mg/L at pH <7 (Deublein et al., 2008). The same concentrations of acetic acid in cyanides samples could indicate inhibition of anaerobic process. Because of the inhibition due to cyanide toxicity, we could assume that hydrolysis, acidogenesis and acetogenesis were inhibited. Therefore methanogenesis could not take place, at least not in full extent as can be seen from Fig. 23. Because of inhibition, there was no acetic acid accumulation during 29 days of incubation.

Most authors report on hydrolytic pathway in anaerobic degradation of CN^- (Fedorak and Hrudey 1989, Paixao et al., 2000, Annachhatre and Amornkaew., 2000, 2001; Kunz et al., 1992; Fallon 1992, Fallon et al., 1991). Reductive pathway is believed to be uncommon and there are no reports to best of our knowledge about occurrence of such pathway under anaerobic conditions since enzyme (nitrogenase) required for such pathway is very rarely found. Enzyme utilizes HCN and produce methane and ammonia as the end products.

Hydrolytic reactions

Cyanide Hydratase	Cyanidase	
$HCN + H_20 \rightarrow HCONH_2$	$HCN + 2H_2O \rightarrow HCOOH + NH_3$	(9)
Nitrile hydratase	Nitrilase	
$R\text{-}CN + H_2O \rightarrow R\text{-}CONH_2$	$R\text{-}CN + 2H_2O \rightarrow R\text{-}COOH + NH_3$	(10)

Reductive reactions

$$HCN + 2H^+ + 2e^- \rightarrow CH_2 = NH + H_2O \rightarrow CH_2 = O$$

$$\downarrow$$

$$CH_2 = NH + 2H^+ + 2e^- \rightarrow CH_3 - NH_2 + 2H^+ + 2e^- \rightarrow CH_4 + NH_3 \quad (11)$$

Regarding to the possible pathways, inhibitors of methanogenesis next to CN^-, could be ammonia, but regarding to the pH in our experiment 7-7.5 and temperature 37°C, ammonia concentration could not inhibit the process. Final concentrations of ammonia in our experiment were 255.6 ± 1.3 and 253.6 ± 3.4 mg/L in case of cyanide samples respectively (Fig. 28). These conditions are well within fully functional conditions as the general inhibitory concentrations start at 1500 mg/L at pH higher than 8 (Deublein et al., 2008).

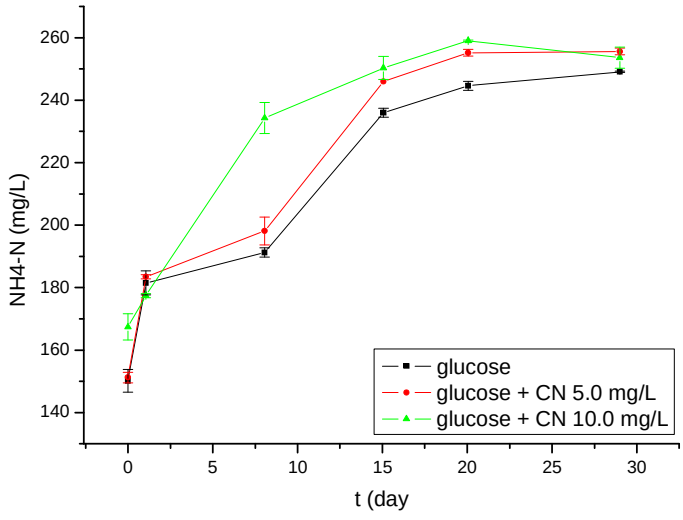

***Fig. 28**: Concentrations of ammonia (NH4-N) during 29 days of incubation. Values are means of three replicates ± SD.*

There was also no significant difference in ammonia concentrations between positive control (249.1 ± 0.3 mg/L) and CN^- amended samples (255.6 ± 1.3 and 253.6 ± 3.4 mg/L respectively), but the process proceeded without inhibition in the case of glucose, meanwhile in case of CN^- methanogenesis was inhibited. These results rule out ammonia as inhibitor of the process in this case.

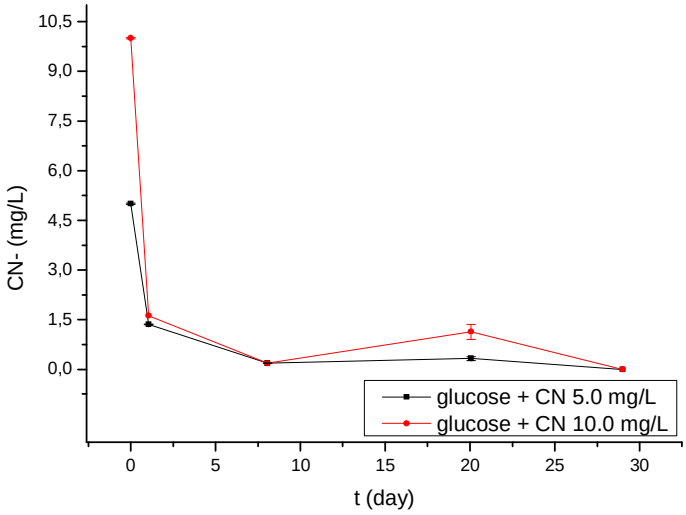

Fig. 29**:** *Concentrations of free cyanide during 29 days of incubation. Values are means of three replicates ± SD.*

Concentration of free cyanide was depleted in 21 days of incubation without any evidence of anaerobic transformation (Fig. 29). Measurements of total cyanide concentrations (TCN) revealed that TCN concentration after 29 days of incubation was almost the same as it was in the beginning of experiment as shown in Fig. 30 and Fig. 31. At the beginning of the experiment (loading rate 10 mg/L of KCN), measured TCN concentration was 10.42 ± 0.09 mg/L and at the end it was 9.36 ± 0.1 mg/L. So, only 1.06 mg/L (10.3 %) of the total CN^- present was removed from the system.

The same results were obtained for the samples amended with 5 mg/L cyanide, where 5.42 ± 0.07 mg/L of TCN was measured at the beginning of the experiment and 4.32 ± 0.09 mg/L of TCN (20,3 % removal) after 29 days of incubation, meanwhile free cyanide decreased from 5 mg/L to under the detection limit (0.01 mg/L).

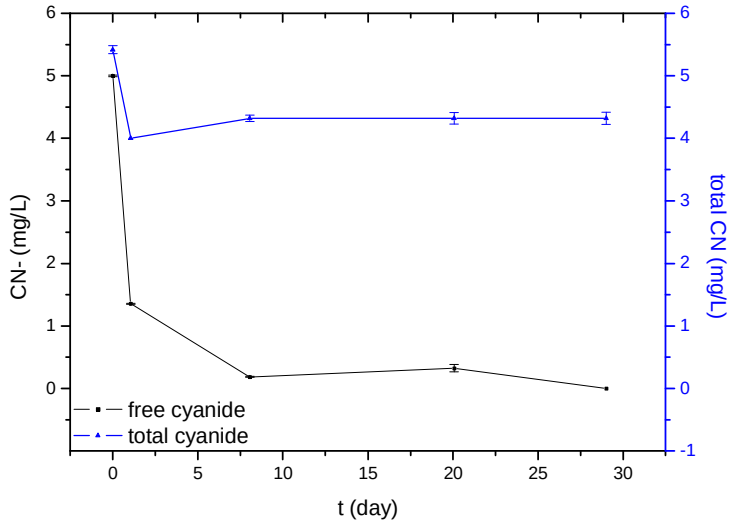

Fig. 30**:** *Concentrations of free cyanide and total cyanide for sample with initial cyanide concentrations 5 mg/L during 29 days of incubation. Values are means of three replicates ± SD.*

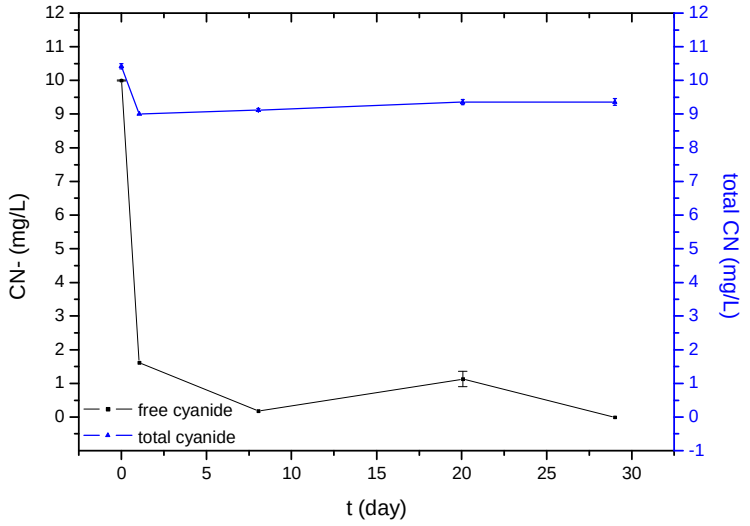

Fig. 31**:** *Concentrations of free cyanide and total cyanide for sample with initial cyanide concentrations 10 mg/L during 29 days of incubation. Values are means of three replicates ± SD.*

Further we analyzed microbial biomass for the presence of metals, which are very common in WWTP sludge (Alonso Alvarez et al., 2002, Hua et al., 2008). Since the simplest form of soluble cyanide is the negatively charged cyanide ion, CN^-, which is composed of a carbon atom triple bonded to a nitrogen atom (C≡N). Because of this bond cyanide anion is a versatile ligand that reacts with many metal cations to form

metal-cyanide complexes (MCC). The results revealed high concentrations of iron and also presence of some other metals as shown in Table 10.

Table 10: *Concentrations of metals present in CSTR microbial biomass*

Metal	Concentration mg/L
Fe	461 ± 15
Ni	1.1 ± 0.1
Zn	52 ± 1
Cu	19.8 ± 0.1

Iron is known to form very strong complexes with cyanide. Based on that knowledge and analytical results we concluded that free cyanide was transformed to metal-cyanide complex. Regarding to the metals present in the analyzed microbial biomass four species of metal-cyanide could be formed; Ni, Cd, Zn, Fe. Since the Cd, Ni and Zn form weak complexes with cyanide, and Fe forms strong MCC and also have the highest equilibrium constant (Table 11) for formation of MCC, we assume that iron-cyanide complex prevails in the system. Schecher et al. (1998) calculated that in anoxic conditions the ferrocyanide form ($Fe(CN)^{4-}_6$) prevails. This is a very strong complex and there are no reports of anaerobic degradation of $Fe(CN)^{4-}_6$. Since the MCC are not biologically available (but still toxic to microbial consortium), dissociation is needed for biological treatment. Dissociation of iron-cyanide complexes is only possible under strong acidic conditions (pH=1.5, heat 125ºC, for 2h; APHA, 2005, ASTM 1998) to dissociate and form free cyanide. Meeussen et al. (1992) demonstrated the high degree stability of ferrocyanide $Fe(CN)^{4-}_6$. Projected half-lives were raging from 1 year under reducing conditions ($pE \approx 5$) at pH 4 to 1000 years at the same pH under oxidizing conditions ($pE \approx 10$). Acidic conditions are toxic for anaerobic microbial consortia, since the optimum pH for methane-forming microorganisms is at 6.7-7.5 (Deublein et al., 2008).

Table 11: *Equilibrium constants for formation of selected metal-cyanide complexes (Schmel g.a. 1989)*

Reaction	Log *K* (at 25ºC, *I* = 0)
$Cd^{2+} + CN^- \rightarrow CdCN^-$	5.32
$Cd^{2+} + 2CN^- \rightarrow Cd(CN)^0_2$	10.37
$Cd^{2+} + 3CN^- \rightarrow Cd(CN)^-_3$	14.83
$Cd^{2+} + 4CN^- \rightarrow Cd(CN)^{2-}_4$	18.29
$Cu^+ + 2CN^- \rightarrow Cu(CN)^-_2$	24.03
$Cu^+ + 3CN^- \rightarrow Cu(CN)^{2-}_3$	28.65
$Cu^+ + 4CN^- \rightarrow Cu(CN)^{3-}_4$	30.35
$Ni^{2+} + 2CN^- \rightarrow Ni(CN)^0_2$	14.59
$Ni^{2+} + 3CN^- \rightarrow Ni(CN)^-_3$	22.63
$Ni^{2+} + 4CN^- \rightarrow Ni(CN)^{2-}_4$	30.13
$Ni^{2+} + H^+ + 4CN^- \rightarrow NiH(CN)^-_4$	36.75
$Ni^{2+} + 2H^+ + 4CN^- \rightarrow NiH_2(CN)^0_4$	41.46
$Ni^{2+} + 3H^+ + 4CN^- \rightarrow NiH_3(CN)^+_4$	43.95
$Zn^{2+} + 2CN^- \rightarrow Zn(CN)^0_2$	11.07
$Zn^{2+} + 3CN^- \rightarrow Zn(CN)^-_3$	16.05
$Zn^{2+} + 4CN^- \rightarrow Zn(CN)^{2-}_4$	16.72
$Fe^{2+} + 6CN^- \rightarrow Fe(CN)^{4-}_6$	45.61
$Fe^{2+} + H^+ + 6CN^- \rightarrow HFe(CN)^{3-}_6$	50.00
$Fe^{2+} + 2H^+ + 6CN^- \rightarrow H_2Fe(CN)^{2-}$	52.45
$Fe^{3+} + 6CN^- \rightarrow Fe(CN)^{3-}_6$	52.63
$2Fe^{2+} + 6CN^- \rightarrow Fe_2(CN)^0_6$	56.98

It was reported that, MCC are less toxic to microbial community than free cyanide (Rollinson et al., 1992). Quan et al. (2004) reported removal of 0.5 mM nickel-complexed cyanide $Ni(CN)^{2-}_4$ under sulfate-reducing conditions. The consortium enriched for cyanide degradation effectively removed nickel-complexed cyanide in 11 days. In another experiment, which lasted 17 days, microbial consortium degraded the free cyanide ion concentration from 0.2 mM to 0.05 mM. However, efficiency was only 20 % regarding to the degradation of nickel-complexed cyanide and this suggests that free cyanide is more toxic in comparison with metal-complexed cyanide.

The microbial biomass was not tested for the presence of MCC, but regarding to the result of TCN and heavy metals we could concluded that there were probably ferrocyanide complexes present in the system, probably $Fe(CN)^{4-}_6$ species, since it was reported that this was prevailing form of ferrocyanide in anoxic conditions

(Schecher et al., 1998). Since the MCC are less toxic than free cyanide we assume that this was probably the reason that in our experiments methanogenesis was still present in comparison to the previous reports of total inhibition, in one case at concentrations 2.0 mg/L of free cyanide (Fedorak and Hrudey 1989) and in the other case at 5.0 mg/L (Gijzen et al., 2000). Poor biological availability of MCC for microorganisms resulted in limited cyanide removal. We could not find evidence of biological transformation of cyanide, since there were no intermediate products of hydrolytic or reductive pathways of cyanide degradation. Small amounts of cyanide missing could be explained either by volatilization as $HCN_{(g)}$ or by biological transformation during the first days of incubation when free cyanide was still available (Sharpe, 1976). $HCN_{(g)}$ was not detected on GC-MS analysis.

From the results we concluded that none of these possible pathways took part in our experiment with 5.0 and 10.0 mg/L cyanide concentrations, which is yet another indicator of process inhibition and also indicator of CN^- toxicity to the microbial community.

Since the Gijzen et al. (2000) reported about adaptation of UASB sludge to cyanide influent concentrations as high as 125 mg/L we tested the adaptivity of microbial consortium to the cyanide toxicity in the next experiments.

Significant findings in the 2nd experiment:

We could not find evidence of anaerobic microbiological transformation of cyanide at concentration 5.0 or 10.0 mg/L in CSTR microbial biomass, since there were no intermediate products of hydrolytic pathways for cyanide degradation detected. Poor biological availability of metal-cyanide complexes (MCC) for microorganisms resulted in minor cyanide removal.

3.3 Experiment III: CSTR sludge amended three times with 2 mg /L of KCN

Aim: Main goal of this experiment was to achieve adaptation of microbial community to the cyanide toxicity since in the 2nd experiment methanogenesis was highly inhibited at concentration of 5.0 mg/L cyanide and contained high concentrations of iron ferrocyanide complexes. In order to achieve methanogenesis without noticeable inhibition at final CN^- concentrations higher than 5.0 mg/L of total cyanide, we used step-wise increasing of free cyanide (KCN) concentrations during 63 days of incubation by three times amendment of 2 mg/L cyanide.

At the beginning of the experiment microbial biomass in the reactors was amended with glucose and 2 mg/L of free cyanide (in the form of KCN). Positive control was amended only with glucose in order to observe the anaerobic process in the absence of cyanide toxicity. In the case of glucose sample (positive control) methanogenesis started immediately, meanwhile in the case of cyanide sample lag phase was observed (Fig. 32). Lag phase lasted for approximately 5 days in cyanide-amended sample with initial concentration 2.0 mg/L of KCN. Following the lag period normal production of biogas was observed.

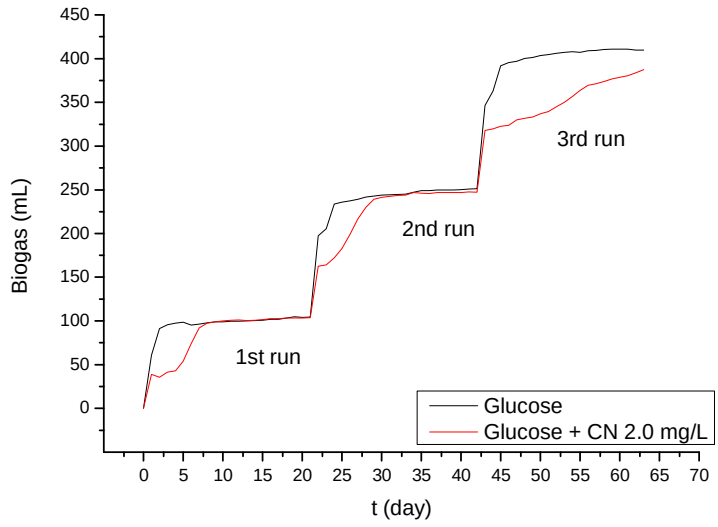

***Fig. 32*:** *Cumulative biogas production during 63 days of incubation. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

After day 13 of incubation only small increment in biogas production as well as in methane content was detected. Methane content for glucose sample was 61.98 ± 1.99 % and 62 ± 1.8 % on day 13 and 21, respectively. The methane content of cyanide amended samples was similar as shown in Fig. 33, 61.9 ± 0.36 % and 63.7 ± 0.04 % on day 13 and 21, respectively. On day 21 samples were again amended with substrates of the same concentration as in the first amendment. As in the first run, biogas production started immediately for the glucose sample. In the case of cyanide amended samples lag phase was observed again. For cyanide amended sample, lag phase was shortened from 5 days in the previous run to 4 days for the second amendment (Fig. 32). Following the lag phase, normal biogas production was observed and cyanide sample reached almost the same level of cumulative biogas production (143.5 ± 1.3 mL) as the positive control (146.9 ± 1.7 mL). Level of cumulative biogas production was higher than in previous run in both samples, where cumulative biogas production was 104.31 ± 3 mL for glucose sample and 103.7 ± 2.9 mL for cyanide sample.

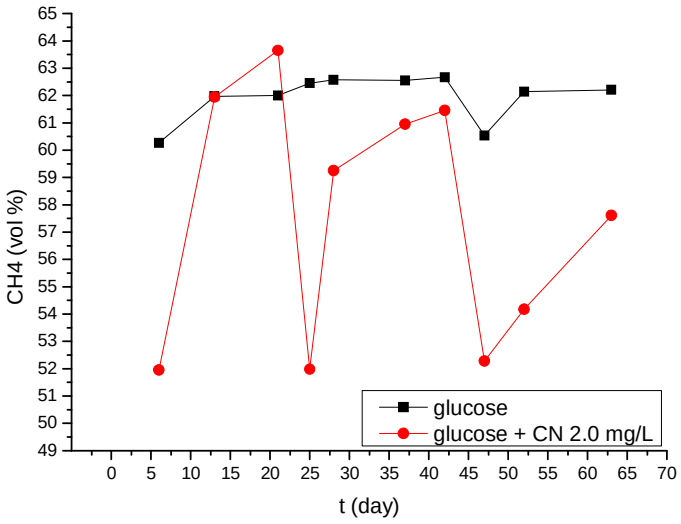

***Fig. 33**: Methane content in produced biogas during 63 days of incubation. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Following the next 21 days (42 day form the beginning of the experiment), inoculum was again amended with the same concentrations of glucose and cyanide. In the case of glucose there were no changes in biogas production in comparison to the first two

runs. In the case of cyanide-amended sample, a longer lag phase occurred than was observed before in the first two amendments and lasted approximately 10 days. This kind of behavior was expected and could be linked to increasing total cyanide concentrations (formation of ferrocyanide complexes), which was 5.7 ± 0.3 mg/L at the end of experiment. Cumulative biogas production in the last run was 140.1 ± 3.5 mL for cyanide sample and 158.6 ± 1.7 mL for glucose sample. Methane content at the end of the third amendment (day 63) was 62.2 ± 3.5 % and 57.6 ± 3.3 % for glucose and cyanide samples respectively.

Concentration of free cyanide was decreasing following each application (days 0, 21 and 42) as shown in Fig. 34. With respect to the results of total cyanide (5.7 ± 0.3 mg/L) at the end of the experiment on day 63, free cyanide was transformed only to the metal-cyanide complexes. Methanogenesis was thus inhibited for only a few days in case of cyanide amended samples. Following this short lag period normal biogas production (regarding to the positive control) was observed.

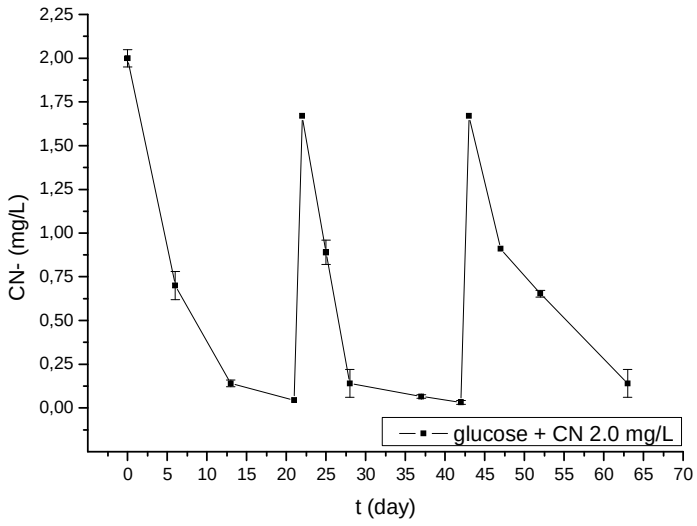

Fig. 34**:** *Concentrations of free cyanide for sample with initial cyanide concentration 2 mg/L during 63 days of incubation. Values are means of three replicates ± SD.*

COD removal efficiency in the first two runs was similar in all samples. The only difference was during the lag phase in case of cyanide samples, where glucose sample showed more efficient COD removal. After the lag phase COD removal was almost identical for all samples as shown in Fig. 35.

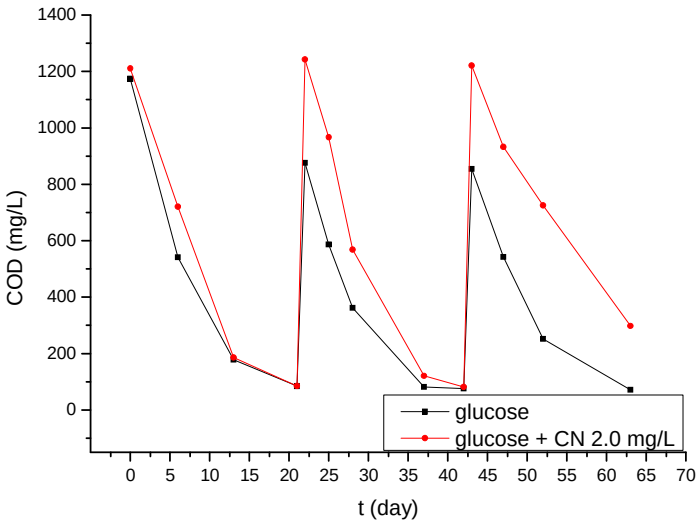

Fig. 35**:** *COD values for adaptation experiment during 63 days of incubation Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

It was reported before (Yang et al., 1980) that following the reduced concentration of free cyanide normal methanogenesis occurred. After the 3rd run significantly lower COD removal efficiency and methane content in biogas were detected for the cyanide-amended samples (Fig. 33) in comparison to the positive control (glucose sample). This was due to the accumulated total cyanide (TCN) in the concentration of 5.7 ± 0.3 mg/L of cyanide-amended sample.

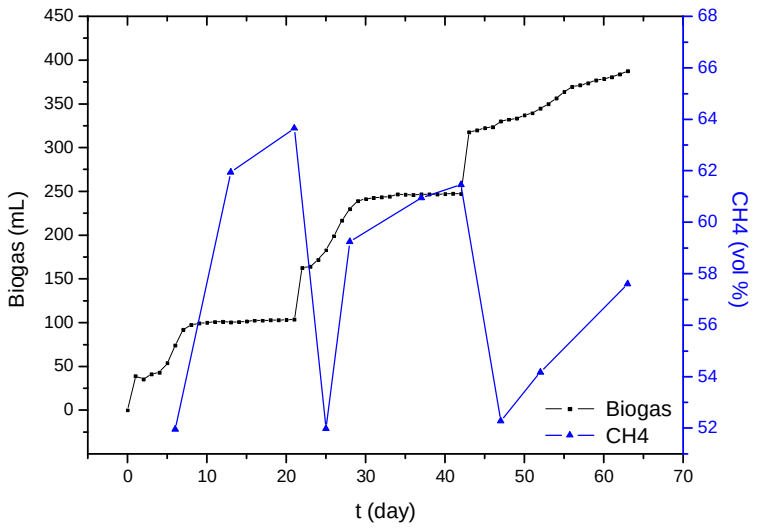

Fig. 36**:** *Cumulative biogas production and CH_4 content for sample with initial cyanide concentration of 2 mg/L during 63 days of incubation. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Free cyanide is more toxic to microbial communities than is cyanide complexed with metal (Rollinson et al., 1987). On Fig. 36 we can observe correlation between lag phases and methane content. We believe that lag phase occurred during the complexation of free cyanide to form metal complexed cyanide. Following the cyanide complexation the toxicity decreases and normal biogas production was observed, comparable to glucose amended positive control. Another indicator for these events is demonstrated on Fig. 37 where we can observe correlation between the lag phase and free cyanide concentrations.

With the decreasing free cyanide concentrations, we can observe the end of lag phase and more biogas produced with higher concentrations of methane content in comparison with methane content during the lag phase. The average methane content during the lag phases was 52.3 ± 0.5 % for cyanide sample. Meanwhile in positive control at the same measurements points, methane concentration was significantly higher 61.2 ± 1.4%. This is another indicator that application of free cyanide to normal performing reactor has an inhibitory effect, but after the metal complexation, normal methanogenesis occurred. Cumulative biogas production during 63 days of incubation for positive control was 409.8 ± 1.7 mL and for cyanide sample 387.3 ± 3.5 mL as seen from Fig. 32.

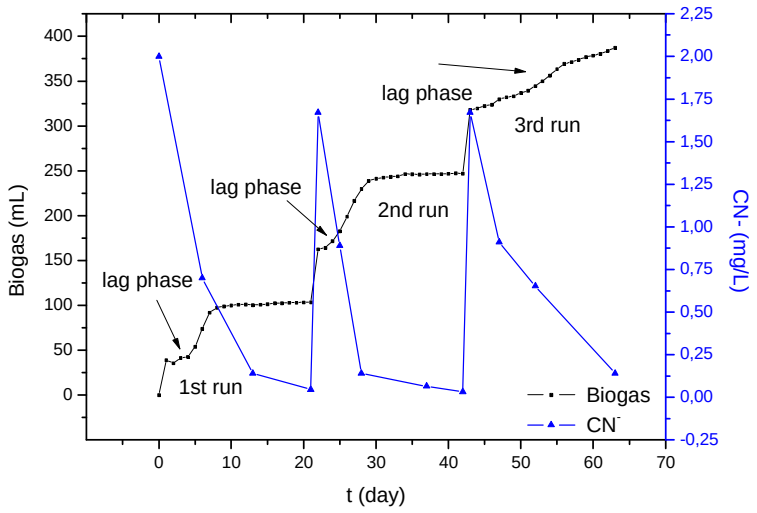

Fig. 37**:** *Cumulative biogas production and CN concentrations for sample with initial cyanide concentration of 2 mg/L during 63 days of incubation. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

If we compare these results with the results of the first experiment where 5.0 mg/L of free cyanide was directly applied, we can observe huge differences in process. When 5.0 mg/L of free cyanide was directly applied methanogenesis was strongly inhibited (Fig. 22, Fig. 23), but when a stepwise increment of cyanide concentration was used we detected a short-term inhibition, in comparison to the positive control. Inhibition was present only during the lag phase and after that normal biogas production was observed again. In both cases only small amounts of cyanide were removed. Most of the cyanide was still present in the final sludge in the form of metal-cyanide complexes, most probably ferrocyanide, regarding to the metals in biomass (Table 10).

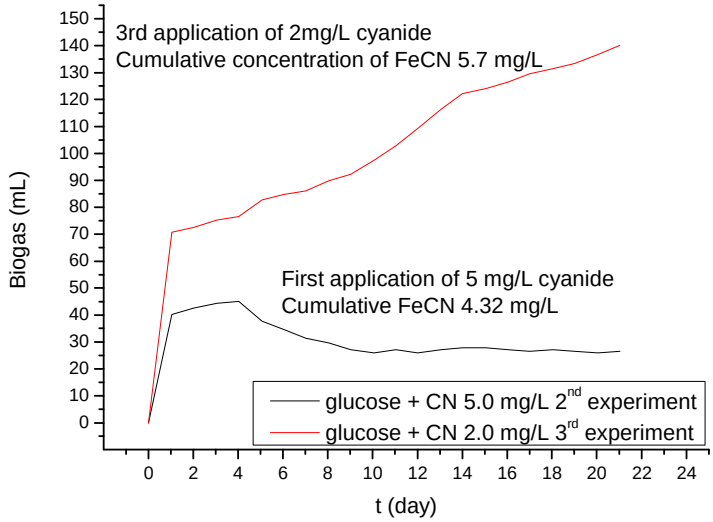

Fig. 38**:** *Cumulative biogas production – comparison between 2nd and 3rd experiment. Values are means of three replicates ± SD. SD is omitted for clarity.*

From Fig. 38 we can observe differences in cumulated biogas between 2nd and 3rd experiment. Final total cyanide concentration in the second experiment was 4.32 ± 0.09 mg/L, and there was 33.3 mL of accumulated biogas with average methane content of 37.4 % ± 1. Meanwhile, at the end of 3rd experiment total cyanide concentration was 5.7 ± 0.3 mg/L, and there was 140.1 ± 3.5 mL of accumulated biogas with methane content (last 21 days of incubation) of 57.6 ± 3.3 %.

There was no COD removal detected in the 2nd experiment, meanwhile in the last run of the 3rd experiment the COD removal efficiency was 75.6 ± 1.7 %.

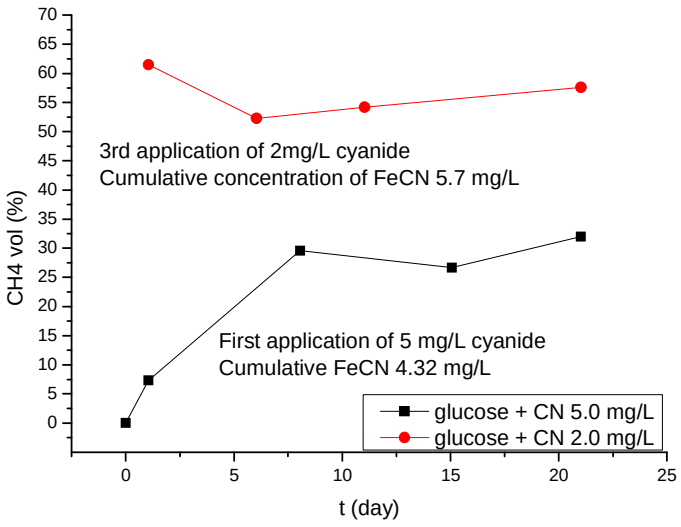

***Fig. 39**: Methane content in biogas – comparison between the 1st and the 2nd experiment. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Toxicity of cyanide to anaerobic microbial consortia has been previously reported (Gijzen et al., 2000, Fallon et al., 1991, Annachhatre and Amornakaew 2001, Smith et al., 1985). Laha and Luthy (1992) reported the tolerance of bacteria to cyanide species at concentrations from 10 to 1000 mg/L, without evidence that bacterial consortium could grow on cyanide as carbon source. At direct application of 5 mg/L KCN (glucose as carbon source), we observed strong inhibition of anaerobic process and there was no cyanide removal and also no COD removal detected. But when we applied a stepwise increasing of cyanide concentrations, starting with concentrations of 2 mg/L of KCN, the anaerobic process was performed normally following the lag phase. We assume that during the lag phase, a complexation of free cyanide to iron-complexed cyanide took place, and after that anaerobic process continued without inhibition. We repeated this for additional two times with addition of 2 mg/L of KCN plus glucose as carbon source for each time, and we always observed the same event: following the lag phase (complexation) normal process was observed in comparison to the positive control. At the last, 3rd application of 2 mg/L of KCN lag phase prolonged (Fig. 32) and we assume that this was due to accumulated cyanide effect. Since, we detected almost none of free cyanide and 5.7 ± 0.3 mg/L of TCN in final samples, we concluded that final media with accumulated metal complexes started to inhibit methanogenesis, but its effect was much lower than effect of the direct

application of 5 mg/L KCN. With respect to the reports of adaptation and tolerance of anaerobic microbial consortia to cyanide (Gijzen et al., 2000, Fallon et al., 1991, Laha and Luthy 1992), we concluded that microbial consortia was adapted to the toxicity of metal-complexed cyanide, but could not use cyanide as a carbon source under tested conditions. In our experiments we added glucose as a carbon source, and once that free cyanide was complexed, microbial consortia used glucose as carbon source and normal methanogenesis took place. Our findings can also be corroborated with studies that have shown that cyanide complexes with iron are most resistant to microbial degradation (James and Boegli 2001), and also the studies, which reported on adaptation of microbial consortia to cyanide toxicity (Gijzen et al., 2000). In described experiments we used CSTR biomass, which contained significant fraction of heavy metals (Table 10). Once the free cyanide was complexed, its toxicity was decreased. We tested that hypothesis with short-term sub-experiment with three different concentrations of free cyanide as shown on Fig. 40.

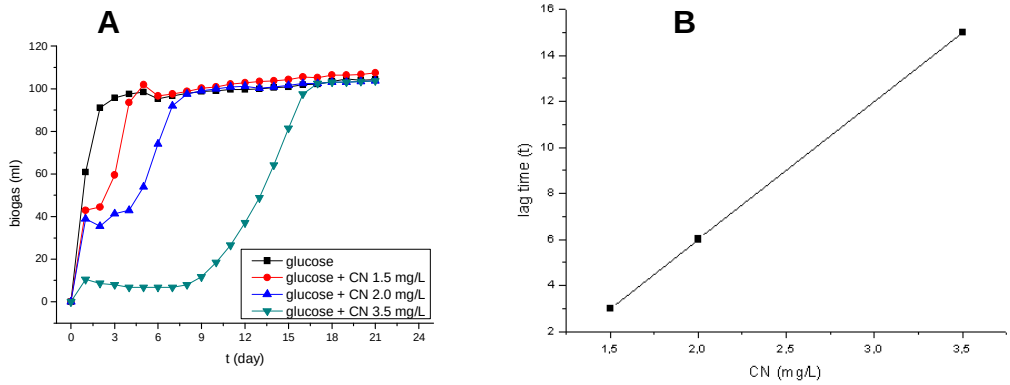

Fig. 40: *Influence of free cyanide concentration on the duration of lag phase in the course of biogas production in laboratory tests of BMP assay (A). The linear relationship between the lag-phase (inhibition) before full biogas production and the concentration of free cyanide in the BMP assay (B).*

From results we can observe a linear correlation between the lag phase and free cyanide concentrations. At the application of free cyanide in concentration of 5 mg/L, the process was strongly inhibited (experiment I), and it never recovered. From these results we can determine that irreversible inhibition point for microbial consortia in selected biomass is between 3.5 and 5 mg/L of cyanide. We believe that complexation takes place during the observed lag phase.

Significant findings the 3rd experiment:

Metal cyanide complexation is probably the main reason that almost none of cyanide was removed during the anaerobic incubation. Up to our knowledge there are no published reports of anaerobic degradation of FeCN. And due to the chemistry of FeCN complexes dissociation (pH 1-2 at 125°C) it is highly unlikely that this is possible under anaerobic conditions. However, our research showed that anaerobic microbial consortia could be adapted to metal-complexed cyanide toxicity, but could still not use cyanide as carbon or nitrogen source for growth.

3.4 Experiment IV: UASB sludge amended with 1, 2, 3 mg / L of KCN

Aim: The aim of this experiment was to apply the microbial biomass that does not contain metals in concentrations that would allow metal-cyanide complexation. In reports on the removal of cyanide under anaerobic conditions, granulated biomass from UASB reactors or synthetic wastewater was used (Fedorak and Hrudey 1989; Gijzen et al., 2000; Fallon et al., 1991; Paixao et al., 2000; Annachhartre and Amornkaew et al., 2000; Chakarobrty et al., 2005). Based on that knowledge we used granulated UASB microbial biomass as inoculum for our next experiments.

Table 12: *Concentrations of metals present in UASB microbial biomass*

Metal	Concentration mg/L
Fe	1.2 ± 0.4
Ni	< 0.5
Zn	1.1 ± 0.1
Cu	0.58 ± 0.2

In this experiment granulated anaerobic biomass from UASB reactor was amended with three different concentrations of cyanide (1.0, 2.0 and 3 mg/L of KCN). Brewery wastewater (BWW) was used as carbon source, because this was the long-term substrate in the bioreactor from which this material was obtained. BMP assay lasted for 21 days. No problems were encountered in completing the process as can be seen from Fig. 41. There was no sign of inhibition or lag phase as witnessed in the previous three experiments with CSTR biomass from wastewater treatment plant.

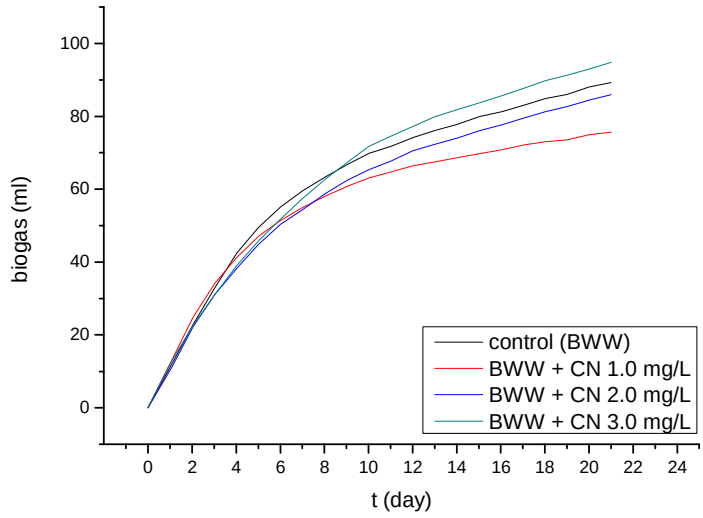

Fig. 41**:** *Cumulative biogas production during 21 days of incubation. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Measurements of biogas composition by GC-MS revealed high methane content in all samples. At the end of experiment, the highest methane content was found in positive control (77 ± 0.2 %) and 74.3 ± 0.9 % in the sample with cyanide concentration of 3.0 mg/L of KCN. No HCN, H_2S or H_2 was detected in the gaseous phase. Cumulative methane production was the highest (71.26 ± 0.9 mL) in the sample with initial cyanide concentration 3.0 mg/L. Meanwhile in positive control cumulative methane production was 68.2 ± 0.3 mL. This could indicate that cyanide was anaerobically converted to methane via hydrolytic pathway as was reported before (Fallon et al., 1991; Fallon 1992, Fedorak and Hrudey 1989). Annachhartre and Amornkaew (2000) reported particularly about hydrolytic pathway at anaerobic degradation of cyanide with biomass obtained from UASB reactor.

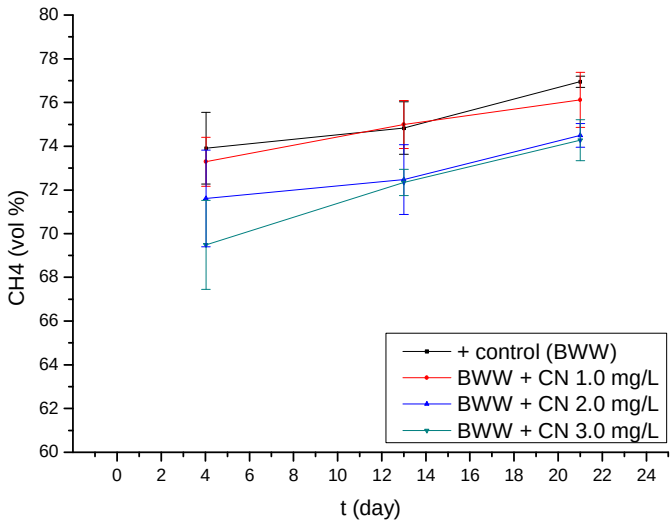

***Fig. 42*:** *Methane content during 21 days of incubation. Values are means of three replicates ± SD.*

Concentration of free cyanide decreased rapidly, during first 5 days of incubation as shown on Fig. 43. After 21 days of incubation concentration of free cyanide stayed almost the same as it was at day 5. In the case of initial concentration 1.0 mg/L KCN there was 78 ± 0.02 % degradation of free cyanide on day 5 and 82.4 ± 0.02 % degradation at day 21. In the case of initial concentration 2.0 and 3.0 mg/L of KCN degradation was 81 ± 0.03 % and 80.5 ± 0.05 % on day 5 and 83,1 ± 0.01 % and 83.5 ± 0.01 % on day 21 respectively. Approximately 83 % degradation of free cyanide was observed in all cases.

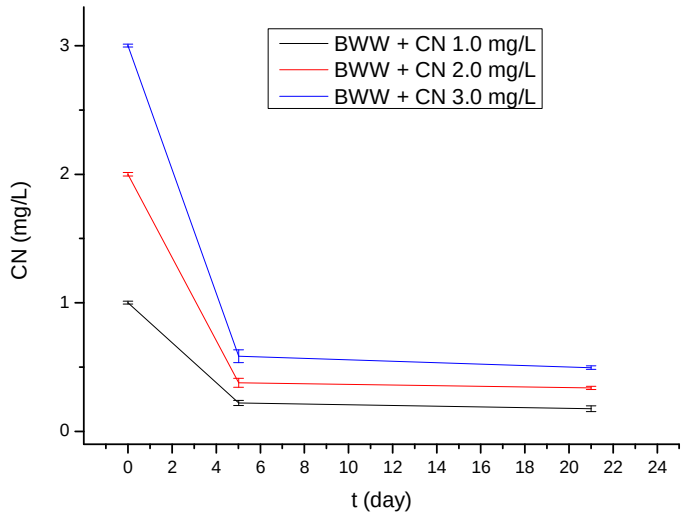

Fig. 43**:** *Concentrations of free cyanide during 21 days of incubation. Values are means of three replicates ± SD.*

Removal of cyanide from the system correlated with cumulative biogas production as seen from Fig. 44. It was reported before, that cyanide degradation without carbon source is not possible (Fallon et al., 1991), and we can conclude that removal of cyanide was directly connected to the availability of carbon source, and once the source was depleted, cyanide concentrations stayed on the same level, simply because there was no energy source for microbial consortia. KCN is also a source of nitrogen, but in very limited amount, which cannot run metabolism of anaerobic microbial consortia.

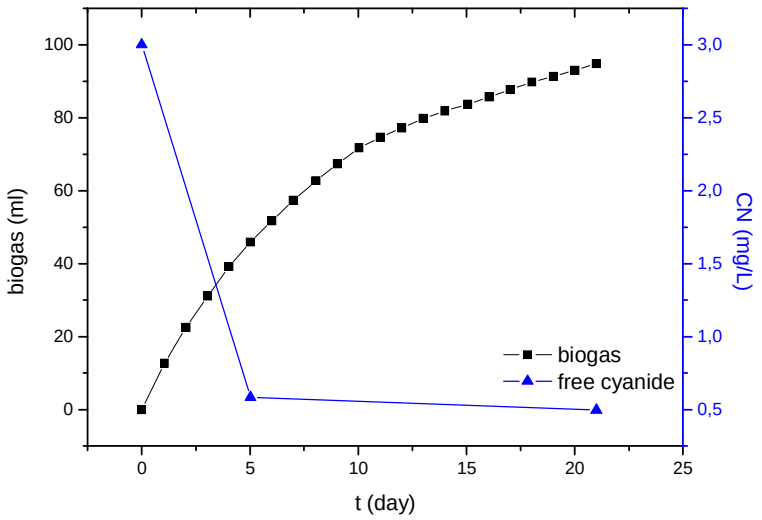

Fig. 44**:** *Concentrations of free cyanide and cumulated biogas for sample with initial cyanide concentration 3mg/L during 21 days of incubation. Values are means of three replicates ± SD.SD is omitted for clarity.*

We failed to measure COD efficiency removal. Calculations revealed that COD efficiency removal was 73.25 ± 1.5 % for positive control, 71.2 ± 1.1 %, 69.32 ± 2.1 %, 72.95 ± 1.9 % for cyanide samples with initial concentrations 1, 2, 3 mg/L respectively. Based on the result of COD removal we also estimated that decreasing the free cyanide concentrations correlated well with COD removal. Concentration of total cyanide after 21 days of incubation for sample with starting concentration 2mg/L was 0.45 mg/L ± 0.1 (free cyanide measurements was 0.338 mg/L ± 0.012.

We also measured concentrations of volatile fatty acids (VFAs, C1-C5 molecules) during 21 days of incubations. On Fig. 45 acetic acid and methane concentrations are shown. Acetic acid is the dominant acid during the process of methanogenesis. If the process is disturbed, this is usually reflected in the concentrations of VFAs. At pH 7, the inhibiting threshold is up to 1000 mg/L of acetic acid (Deublein et al., 2008). In our case the process ran smoothly, and there was no accumulation of VFAs. Comparison between positive control sample and cyanide sample in Fig. 45 shows, that there was no inhibition by cyanide regarding to the positive control.

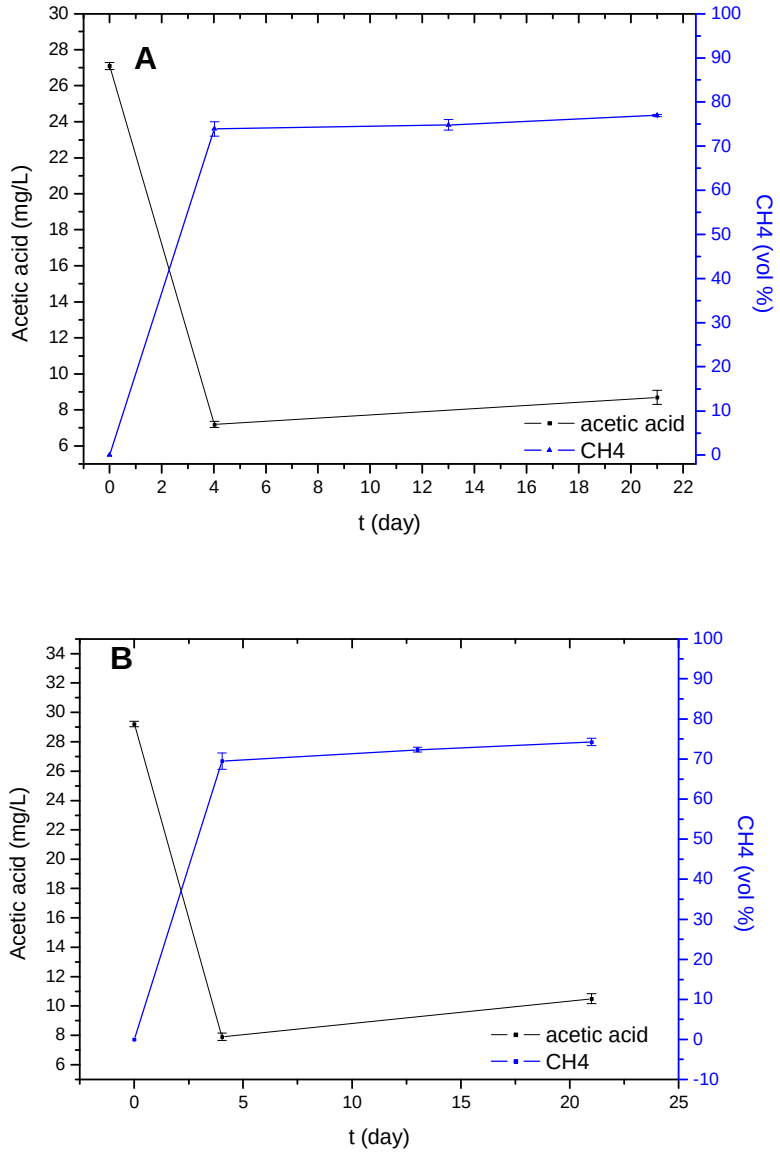

Fig. 45**:** *Concentrations of acetic acid vs. methane content for positive control (A) and cyanide sample with initial concentration 3mg/L of KCN during 21 days of incubation (B). Values are means of three replicates ± SD.*

Significant findings of the 4th experiment:

First experiment with granulated microbial biomass from UASB reactor showed more than 80 % of 3 mg/L free cyanide removal. Measurements of total cyanide revealed that there was no formation of metal-cyanide complexes. This was expected since in selected biomass there were only small amounts of heavy metals Table 12). Higher cumulative methane production in the cyanide sample

(compared to positive control) could indicate that cyanide was anaerobically converted to methane via hydrolytic pathway.

3.5 Experiment V: UASB sludge amended 5 and 8.5 mg / L of KCN

Aim: After the experiment of anaerobic methanogenic degradation with three different concentrations of free cyanide, we tested the effects of higher concentrations of cyanide on UASB microbial biomass. This experiment was conducted in the same way as the 4th experiment except that higher cyanide concentrations (5.0 and 8.5 mg/L) and longer time of incubation (60 days) were used.

Samples amended with initial concentration of 5.0 mg/L of KCN showed no inhibition; meanwhile sample with concentration of 8.5 mg/L of KCN was strongly inhibited as shown in Fig. 46. Inhibition lasted approximately 22 days without significant biogas formation. The only biogas production was observed during the first two days (4.3 mL ± 0.6 and 1.64 mL ± 1 respectively), probably due to delayed toxic effect of cyanide. After day 22, biogas started to accumulate very rapidly and reached the same volume as in 5.0 mg/L at day 38.

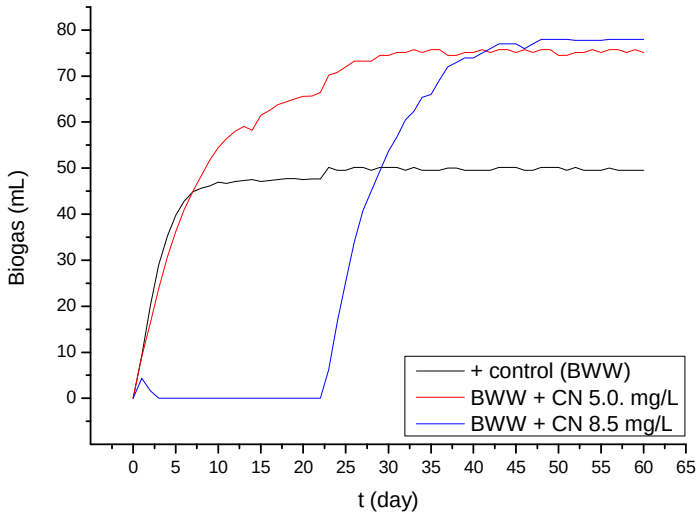

***Fig. 46**: Cumulative biogas production. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Methane content during 60 days of incubation was almost the same in positive control as it was in cyanide sample with concentration 5.0 mg/L of KCN. There was only a small amount of methane detected in cyanide sample with initial KCN concentration of 8.5 mg/L. Methane was probably formed during the first two days of incubation, when there was some small biogas accumulation.

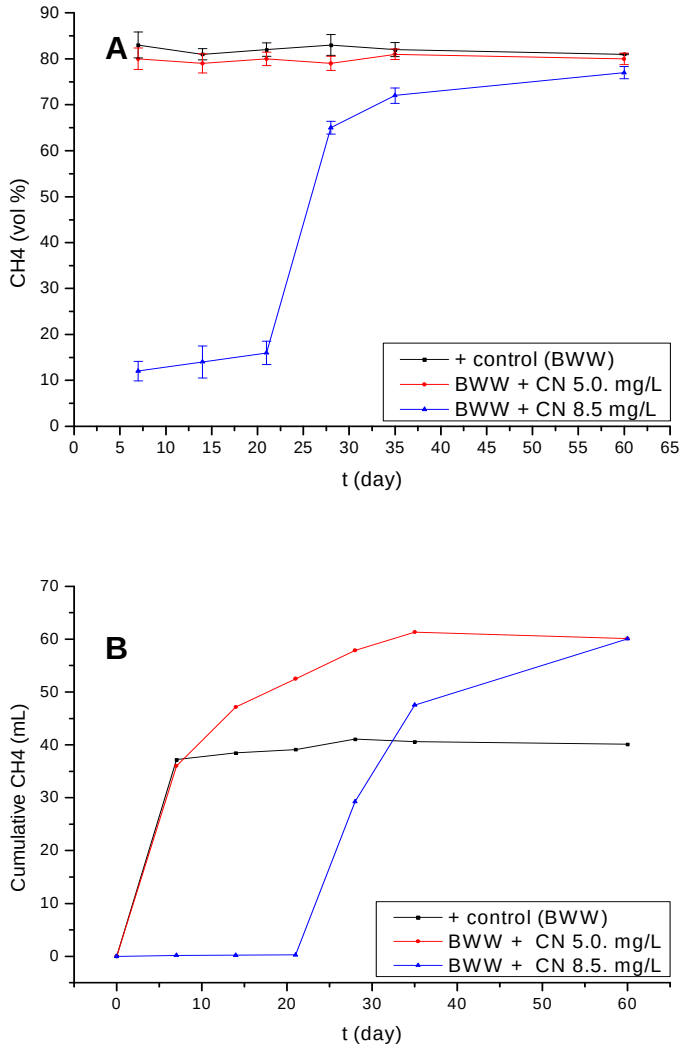

***Fig. 47*:** *Methane content (A) and cumulative methane production (B).Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

Interestingly, the volume of accumulated biogas from cyanide-amended samples was higher than the volume of accumulated biogas from positive control that received only brewery wastewater as carbon source. That was also observed in experiment IV

(Fig. 41), where cyanide sample with initial concentration of 3 mg/L of KCN, also resulted in more accumulated methane compared to positive control.

Methane content at the end of process was almost the same in all samples; meanwhile cumulative methane production was higher in cyanide-amended samples, suggesting that a fraction of biogas was derived from CN^- degradation probably by hydrolytic pathway. From the cumulative methane production (Fig. 47 b) it is evident that more methane was formed in cyanide amended samples. Hydrolytic pathways have several advantages for detoxication of cyanide, because direct enzymatic cleavage of the carbon nitrogen triple bond of HCN serves to degrade the cyano group and thus eliminates the possibility of further reactivity (Raybuck, 1992). The products of hydration (formamide – amide derived from formic acid) and hydrolysis (formic acid and ammonia) are considerably less toxic than cyanide and can serve as carbon and nitrogen source for microbial community. Another advantage is that no additional cofactors are needed; the only requirement for catalysis is presence of functional enzyme (e.g cyanide hydratase, cyanidase, nitrilase, nitrile hydratase). Therefore, hydrolytic systems are adequate for enzyme stabilization and immobilization technology, which can result in the treatment of higher concentrations of cyanide, than could be tolerated by the living cell (Raybuck, 1992). Sanchez et al., (1967) reported of spontaneous abiotic hydrolysis of cyanide to formate, which is thermodynamically favorable reaction.

$$HCN + 2H_2O \rightarrow HCOOH + NH_3 \qquad \Delta G° = -15.6 \text{ kcal} \qquad (12)$$

Fallon (1992) also observed direct hydrolysis of cyanide to formate and bicarbonate. Therefore, cyanide is degraded through conversion to formate and then to CO_2 by formate hydrogenase (Wu et al., 2003). Conversion to formate could be mediated by nitrilase or alternatively to formamide by cyanide hydratase and formamidase (Gupta et al., 2009).

Cyanide Hydratase	Cyanidase	
$HCN + H_20 \rightarrow HCONH_2$	$HCN + 2H_2O \rightarrow HCOOH + NH_3$	(13)
Nitrile hydratase	Nitrilase	
$R\text{-}CN + H_2O \rightarrow R\text{-}CONH_2$	$R\text{-}CN + 2H_2O \rightarrow R\text{-}COOH + NH_3$	(14)

It is well known that anaerobic microbial community converts formate to CO_2 and methane. CO_2 also exists in equilibrium with bicarbonate (HCO_3^-), which is poorly soluble in lipid membranes compared to carbon dioxide; carbon dioxide can freely diffuse in and out of the cell, while bicarbonate must be actively transported (McRae et al, 1983). Further, it is also well known, that in the case of hydrogenotrophic methanogenesis, methanogenic archaea use hydrogen (and also formate) as electron donor to reduce CO_2 to methane. On the other hand formic acid represents viable substrate for methanogens and could play important role in maintaining thermodynamically favorable conditions for cyanide removal, since methanogens utilize formic acid for their own metabolism and thus shifting the equilibrium in the forward direction for hydrolytic reaction (equation 12) with enzyme cyanidase and further initiating cyanide degradation.

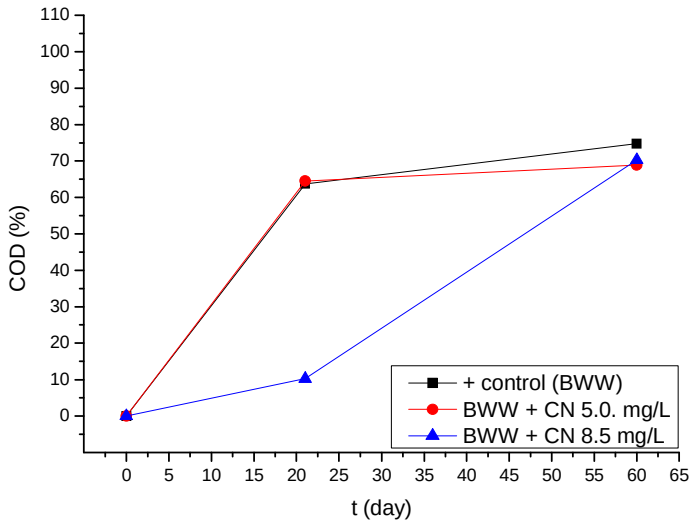

***Fig. 48**: COD efficiency removal during 60 days of incubation.. Values are means of three replicates ± SD.*

COD removal (Fig. 48) for positive control was 74.75 ± 1.4 %, for cyanide sample with initial concentration 5.0 mg/L 68.9 ± 2.1 % and for cyanide sample with initial concentration 8.5 mg/L COD removal was 70.25 ± 0.9 %.

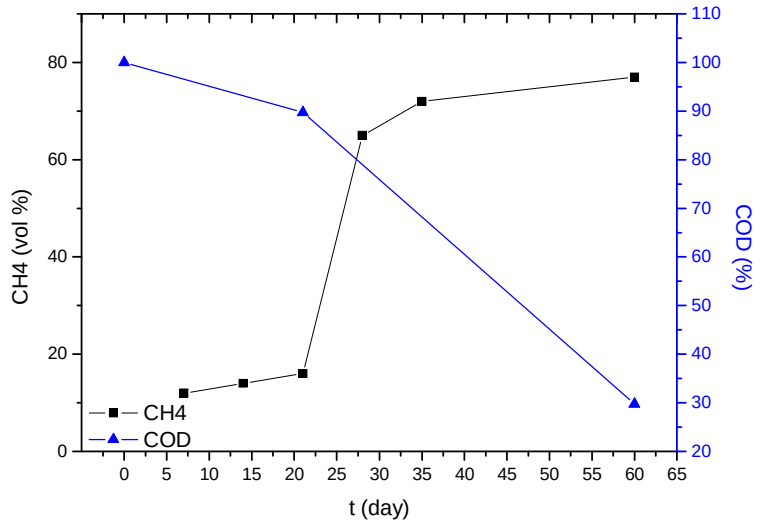

Fig. 49**:** *COD removal efficiency and methane content for sample with initial cyanide concentration 8.5 mg/L during 60 days of incubation. Values are means of three replicates ± SD. SD is omitted for clarity but represented less than 15% of the average.*

There was a delay in COD removal for the highest cyanide concentration as shown on Fig. 49. With the decreasing COD values methane content in accumulated biogas started to rise.

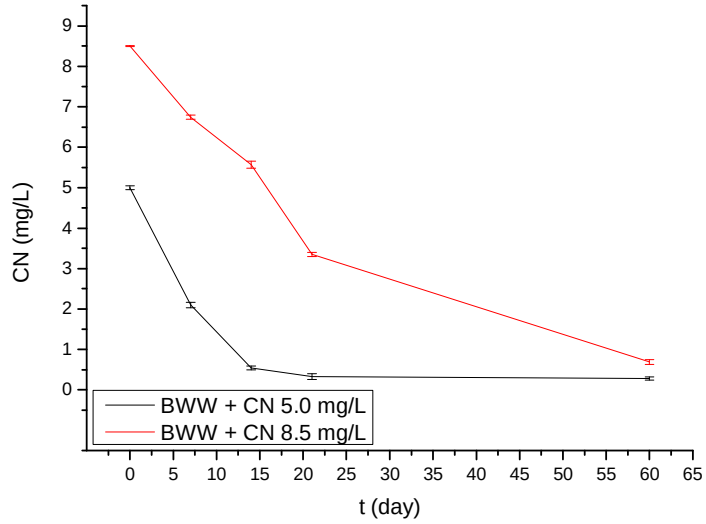

Fig. 50**:** *Concentrations of free cyanide during 60 days of incubation. Values are means of three replicates ± SD.*

Free cyanide concentrations started to decrease immediately in samples amended with initial concentration of 5.0 mg/L. Gijzen et al. (2000) reported strong inhibition of methanogenesis and COD removal at direct application of 5.0 mg/L of KCN to well functioning UASB reactor. We observed perfect functioning of the process. COD removal was almost the same as it was for the positive control. We also observed strong inhibition in samples amended with 8.5 mg/L until the concentration of free cyanide decreased under 4.0 mg/L. After that biogas and methane content started to increase at approximately the same rate as in the control samples (Fig. 47 A). In the case of cyanide samples supplemented with initial concentrations of 5 and 8.5 mg/L of KCN, 94.34 ± 0.043 % and 93.1 ± 0.061 % of cyanide were removed, respectively. Total cyanide analyses of sludge revealed that removal of cyanide was not due to complexation with metals.

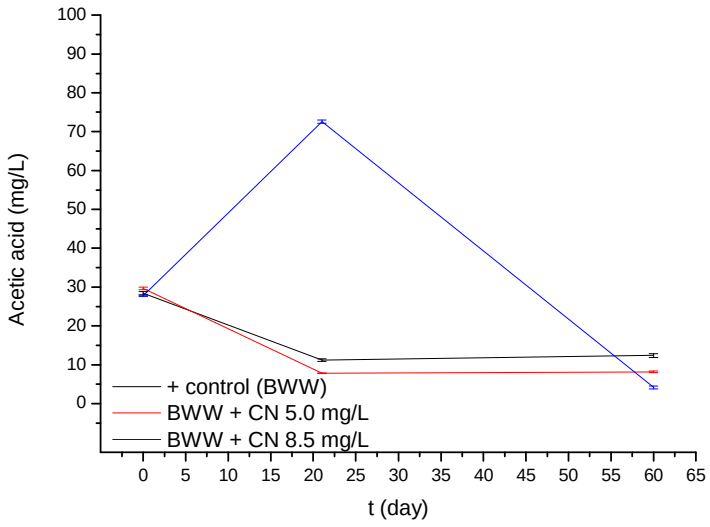

Fig. 51**:** *Concentrations of acetic acid in samples during 60 days of incubation. Values are means of three replicates ± SD.*

Concentrations of acetic acid were almost the same in all samples at the beginning of incubation (t_0). In bioreactors with initial concentrations of 8.5 mg/L of KCN, acetic acid started to accumulate. The concentrations of acetic and propionic acid were 72.6 ± 0.4 mg/L and 3.9 ± 0.2 at day 21 (not shown on Fig. 51). The positive control and 5.0 mg/L cyanide amended sample that contained 11.2 ± 0.3 and 7.8 ± 0.1 mg/L of acetic acid, respectively, whereas the propionic acid was below the detection limit. From these observations, we concluded that methanogenesis was inhibited for the first 21 days in case of the sample with initial concentration of 8.5 mg/L KCN, until the concentration of free cyanide decreased under 4.0 mg/ L (Fig. 50). Then normal methanogenesis was observed as seen from figure Fig. 46 and Fig. 47.

Concentrations of NH_4-N were 172.6 times lower than in CSTR biomass experiments due to different properties of microbial biomass. As seen from figure 52, concentrations of ammonia were almost the same at the end of incubation time in positive control and cyanide amended sample with initial concentration of 5.0 mg/L of KCN. In case of cyanide sample with initial concentration of 8.5 mg/L of KCN, NH_4-N concentrations were 1.72 times higher. Differences in NH_4-N concentrations between control and cyanide amended samples were thus too small to serve as evidence for the presence of enzymes involved in cyanide transformation via hydrolytic pathway. In general, when NH_4-N concentrations are of similar

concentration as observed in our experiments, the ammonia derived from cyanide degradation could be completely consumed in the process of cell synthesis (Raybuck 1992).

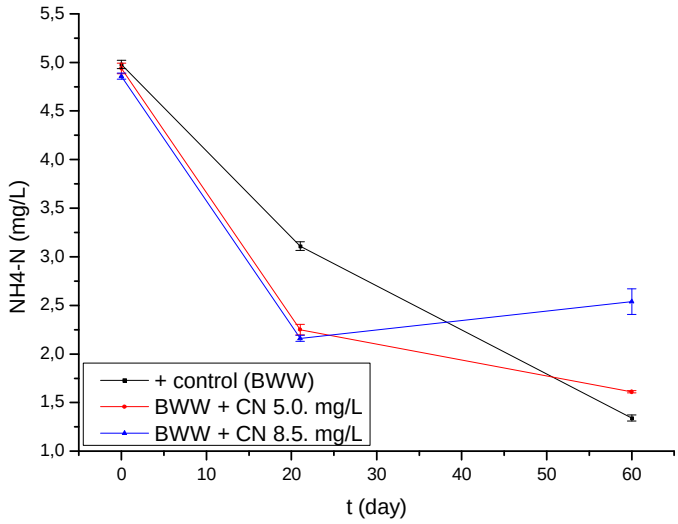

Fig. 52: *Concentrations of ammonia (NH4-N) during 60 days of incubation. Values are means of three replicates ± SD.*

Measurements of total cyanide at the end of incubation time showed that there was no complexed cyanide present in the sludge. In addition, no HCN was detected by analyses of gaseous phase. With respect to the COD removal and methane content measured in our experiments, we believe that cyanide was degraded into simple organic or inorganic molecules (abiotic), which were further metabolized by enzymes of microbial community (biotic) to ammonia and either carbon dioxide or methane as was reported before (Fedorak and Hrudey 1989; Gijzen et al., 2000; Fallon et al., 1991; Paixao et al., 2000; Annachhartre and Amornkaew et al., 2000, 2001; Chakarobrty et al., 2005).

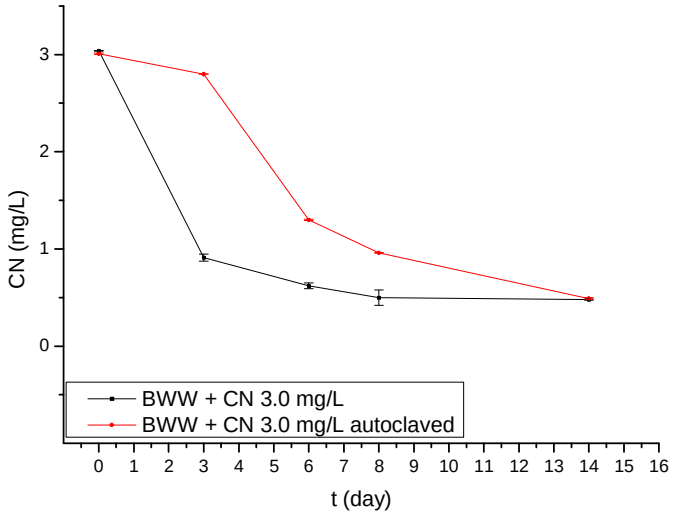

Fig. 53**:** *Concentrations of free cyanide during 14 days of incubation. Values are means of three replicates ± SD.*

In order to determine whether the process of cyanide degradation observed in our experiments was biotic or abiotic we decided to perform **the second (sterilization) subexperiment** in which we applied KCN in the concentration of 3.0 mg/L. This experiment was conducted essentially in the same way as described in materials and methods for experiment IV with an additional step of sterilization of the whole setup by autoclaving. KCN was added aseptically after autoclaving to prevent its degradation during the process of sterilization. As shown in Figure 54, the concentration of free cyanide decreased faster in the sample, which was not autoclaved and thus contained live microbial biomass. After 14 days of incubation the final concentration of free cyanide was the same in both samples. GC-MS measurements revealed that CO_2 and N_2 were the sole major constituents of the gas phase of autoclaved samples with traces of other gases below detection limit. The non-autoclaved samples contained 78.2 ± 0.6 % of methane, which was similar to the methane content in samples amended with 3 mg/L cyanide in experiment IV. The measurements of NH_4-N at day 3 of the experiment V showed the presence of 2.2 ± 0.1 mg/L NH_4-N in autoclaved sample, 50.3 ± 0.3 mg/L in positive control and 75.2 ± 0.3 mg/L in cyanide-amended sample. At end of experiment concentrations of NH_4-N were 4.2 ± 0.4 mg/L for autoclaved sample, 5.1 ± 0.2 mg/L for positive control and 7.3 ± 0.1 mg/L for cyanide-amended sample. This is yet another evidence for the microbial nature of cyanide degradation in our experiment. Five

enzymes, which are present in microbial systems, catalyze the hydrolytic pathway: (i) cyanide hydratase, (ii) nitrile hydratase, (iii) carbonyl pathways (thiocyanate hydrolase), (iv) nitrilase, and (v) cyanidase. The first three enzymes are known to possess sufficient substrate specificity to directly hydrolyze and cleave the carbon–nitrogen triple bond to form formamide. The latter two convert the resulting formamide further to ammonia and methanoic or formic acid, which are utilized in microbial metabolism (Gupta et al., 2010). Our results showing low ammonia concentrations also indicate that ammonia derived from cyanide degradation was most probably taken up by microorganisms for the synthesis of cell constituents. In the experiment V with 5 and 8.5 mg/L we analyzed NH_4-N only at day 21 and day 60 and probably missed the most informative ammonia formation window.

These results described above indicated two things: (i) the amended cyanide is spontaneously transformed via abiotic hydrolytic pathway yielding formic acid and ammonia in sterile conditions as was reported before by Fallon (1992):

$$HCN + 2H_2O \rightarrow HCOOH + NH_3 \quad (15)$$

(ii) with the respect to faster turnover of free cyanide in presence of biotic factors we could conclude that UASB microbial consortia actually cleaved carbon nitrogen triple bond of HCN using their enzymes and then metabolized the product of hydration (formamide) and hydrolysis (formic acid and ammonia and either carbon dioxide or methane).

Significant findings the 5th experiment:

UASB microbial biomass was successful in degradation of cyanide without noticeable inhibition up to 5 mg/L of KCN. At higher initial concentration of 8.5 mg/L of KCN lag phase was detected, which lasted 21 days. Following the lag phase normal methanogenesis at comparable rates as observed in positive controls was observed. There was more accumulated biogas and methane after 60 days of incubation in cyanide samples in comparison to the positive control, suggesting that cyanide was anaerobically transformed. Measurements of the total and free cyanide in microbial sludge and biogas revealed that none of cyanide was complexed or escaped via the gas phase. Cyanide was removed

from the system regardless the presence of biotic factors, however, abiotic conversion proceeded at two times lower rates. Based on the results of the study we concluded that anaerobic degradation of cyanide observed in our experiment was most probably a combination of simultaneous abiotic and biotic.

4 CONCLUSIONS OF CHAPTER 3

In our five experiments and three sub experiments we tested two different anaerobic biomasses for cyanide removal under anaerobic conditions. First microbial biomass was from CSTR anaerobic reactor (2^{nd} stage of waste water treatment plant). The diversity of substrates fed to the biomass, occasional presence of cyanides in wastewater and suspended nature had no positive effect on the performance of anaerobic cyanide degradation. Because of the nature of wastewaters the CSTR biomass was full of metals (Table 10), which are known that forms weak and strong metal complexes with cyanide anion. In the selected biomass iron prevailed as transition metal, which forms especially strong complexes as shown in Table 11. There were four possibilities of cyanide degradation in this study: (i) Reductive reactions in which $HCN_{(aq)}$ is converted to methane and ammonia; (ii) Hydrolytic reactions, which can be spontaneous (abiotic) or catalyzed by microbial community enzymes (biotic), forming formamide, formate (converted to bicarbonate) and ammonia; (iii) metal complexation and (iv) also Kiliani–Fischer synthesis. The latter (Kiliani-Fischer synthesis) is the so called reaction between the reducing sugars and cyanide. It was reported by Hope et al. (1991), that degradation of cyanide under anaerobic conditions in the presence of reducing sugars was independent of microbial metabolism. All the sugars tested showed ability to cause the formation of ammonia in the presence of potassium cyanide. We have also performed a control experiment with glucose and KCN, together with negative control (no glucose added). This **third (Kiliani-Fischer) subexperiment** was conducted in the same way as all other experiments with cyanide except that microbial biomass (biotic source) was omitted and the reactions assembled under sterile conditions. We observed cyanide disappearance in all samples, whether glucose was or was not present. Measurements on the GC-MS did not show any formation of gaseous HCN, and we also did not observe any additional formation of ammonia either in gaseous or

aqueous phase. We concluded that under our experimental conditions Kiliani-Fischer synthesis could be excluded.

Which of these reactions will actually take place depends on the kinetic and thermodynamic factors. The basic differences between the CSTR and UASB samples were summarized in Table 13. In the 1st, 2nd, 3rd experiment we observed metal-cyanide complexation. Once the cyanide is complexed (during lag phase) it becomes biologically unavailable or poorly available, which indicates minor removal of cyanide in the 2nd cyanide experiment. Although, that cyanide is complexed and biologically unavailable for degradation it is still toxic for microbial consortium as shown in the 2nd experiment. But it is obvious and was also reported before (Gijzen et al., 2000) that microbial consortia can adapt to toxicity of cyanide to the certain concentrations as was shown in the 3rd experiment when step wise increase of cyanide concentrations was applied. After three successive applications of KCN metal cyanide complexation was detected and final concentration of complexed cyanide after 63 days of incubation was 5.7 ± 0.3 mg/L. Although, that concentration of complexed cyanide was higher then in 3rd experiment (4.32 ± 0.09 mg/L) methanogenesis ran smoothly in comparison with the direct application of 5 mg/L of KCN (Fig. 38). From the results of our experiment we do not believe in the possibility of cyanoferrate degradation under anaerobic conditions or in presence of sulfate reducing bacteria, due to the parameters that are needed for dissociation of the complexes (pH 1-2, temperature 125°C). However, our experiments with suspended biomass from CSTR showed that anaerobic microbial consortia could be adapted to tolerate toxicity of metal-complexed cyanide but could still not be able to use cyanide as carbon or nitrogen source for growth.

On the other hand, granulated nature of UASB microbial biomass and small amounts of metals (Table 12) most probably allowed biotic cyanide degradation. The second microbial biomass that was tested with cyanide substrate originated from industrial well performing UASB reactor (4th and 5th experiment). In this case we actually observed cyanide removal up to 8.5 mg/L of initial concentrations. Up to 5 mg/l of KCN added we did not observe any disturbance of methanogenesis in comparison with positive control. Analyses on all levels showed that no cyanide was complexed or escaped via gas phase. This is probably due to a granulated biomass, which differs

from suspended sludge. Granulated biomass has a layered structure. McLeod et al. (1990) demonstrated that the granular aggregates are three-layered structures. The exterior layer of the granule contained a very heterogeneous population that included acidogens, hydrogen consuming organisms and filaments of various sizes. The middle layer consisted of a slightly less heterogeneous population than the exterior layer. The third layer formed the internal core of the granules. It consisted of large numbers of methanogen *Methanothrix* like cells.

Table 13: Differences in the performance of biomasses in the 5 experiments

parameter	WWTP	Industrial
Reactor type	CSTR	UASB
Biomass	suspended	granulated
Substrate	diverse	brewery waste water
CN- loading	occasional	none
Metals	> 550 mg/L	< 3.5 mg/L
Treating CN- [mg/L]	up to 3.5	up to 8.5
Adaptation	possible	existing activity
Bacterial community	unknown	unknown
Archaeal community	unknown	unknown

Studies on the micro-morphology of the granules demonstrated that colonies of acetogenic bacteria are closely linked with micro-colonies of hydrogenotrophic methanogenic archaea allowing an efficient interspecies hydrogen transfer and as a result, high degradation rates of various substrates (Hulshoff Pol et al., 2004). From this we can also conclude, that granulated biomass consists of thicker biofilms and it is supposed to be capable of handling higher cyanide concentrations due to the minimal exposure of inner biofilm layer. Forming of biofilm can also explain more effective cyanide degradation in batch experiments (Gijzen et al., 2000, Fedorak and Hurdey 1989) in comparison with continuous systems in which biofilm is hardly formed because of continuous flow.

With respect to the experimental data including short-term aseptic experiment we believe that cyanide was actually removed from the system by the hydrolytic reactions, probably partly spontaneous, abiotic and partly biotic with enzymes derived from microbial community. This resulted in the formation of simple organic or inorganic molecules, which were further metabolized by microbial consortia as was reported before (Fedorak and Hrudey 1989; Gijzen et al., 2000; Fallon et al., 1991; Paixao et al., 2000; Annachhartre and Amornkaew et al., 2000; Chakarobrty et

al., 2005). Based on the results of the study we concluded that anaerobic degradation of cyanide is combination of simultaneous abiotic and biotic processes and not exclusively biological.

CHAPTER 4

CHAPTER 4 - MICROBIAL COMMUNITIES IN THE EXPERIMENTS I-V

1 INTRODUCTION

Based on the physiological results obtained in Chapter 3, analyses of the microbial communities at the level of bacteria and archaea were conducted. In order to obtain as much information as possible, microbial communities were analyzed in a number of samples using different but complementary approaches. These are summarized and integrated with the five experiments from Chapter 3 in Table 9.

Table 14: Overview of all experiments showing those that were used for molecular analyses.

	KCN concentration	Biomass	Profiling of microbial communities	Cloning and sequencing	Microarray
Experiment 1	1.5 mg/L	Suspended CSTR			
Experiment 2	5 and 10 mg/L	Suspended CSTR			
Experiment 3	adaptation experiment: 2 mg/L applied 3 times *(1.5, 2 and 3.5 mg/L sub experiment)	Suspended CSTR	T-RFLP bacteria archaea	1 archaeal library 1 bacterial library	3 array samples
Experiment 4	1, 2, 3 mg/L	Granulated UASB			
Experiment 5	5 and 8.5 mg/L (Sterilization sub experiment) (Kiliani-Fischer sub experiment)	Granulated UASB	T-RFLP bacteria archaea	1 archaeal library 2 bacterial libraries	5 array samples

2 MATERIALS AND METHODS

2.1 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP was used to determine the overall similarity of bacterial and archaeal microbial communities in the initial and final samples from incubation experiments with cyanide (Table 14). When the differences between the initial and final microbial communities were found to be significantly different, both communities were used for preparation of clone libraries of target 16S rRNA genes order to identify the key players responsible for biotic cyanide degradation.

Genomic DNA was extracted using UltraClean Soil DNA Isolation kit (MOBIO) according to the manufacturer instructions for maximum DNA yield. The quality of DNA was checked by 1 % agarose gel electrophoresis. PCR amplification was performed using MyCycler (BIO-RAD laboratories). The PCR primers 6-FAM labeled 109f (6-FAM, 5' - CAN GCT CAG TAA CRC GYR-3') and 691r (5'-CGA TTA CAR GAT TTC AC-3') were used to amplify the 16S rRNA gene of methanogenic archaeal community (Watanabe et al., 2004). The PCR primers 6-FAM labeled 27f (6-FAM 5' - AGA GTT TGA TCC TGG CTC AG-3'), 926r (5'-CCG TCA ATT CCT TTR AGT TT-3'), were used to amplify the 16S rRNA gene of methanogenic bacterial community. Three replicate PCR reactions were performed for each DNA extract and subsequently pooled essentially as described before (Stres et al., 2008) PCR program for methanogenic archaeal community was; the initial denaturation (5 min 94°C), and a total 35 cycles, each including 30 s at 94°C, 45 s at 52°C, and 90 s at 72°C, was followed by a final extension step of 10 min at 72°C. PCR program for bacterial community was; the initial denaturation (5 min 94°C), and a total 25 cycles, each including 30 s at 94°C, 45 s at 52°C, and 90 s at 72°C, was followed by a final extension step of 7 min at 72°C. PCR products were examined by electrophoresis on 1.5 % agarose gels. Residual primers were removed using High Pure PCR Product Purification Kit (Roche) according to manufacturer instructions.

To provide a rationale in choosing gene-primers-enzyme combinations with highest resolution in T-RFLP analysis, all restriction enzymes (http://rebase.neb.com/rebase/rebase.html) were tested on 120.000 archaeal sequences (Ribosomal Database Project II (http://rdp.cme.msu.edu/) Release10.05) using BESTRF software as described before (Stres et al., 2009).

T-RFLP analysis was conducted essentially as described before (Stres et al., 2009). Digestion of 50 ng of PCR products was carried out in a 30 μL volume using 3 μL of yellow buffer, 0.5 μL of HhaI and 3 μL of red buffer, 0.5 μL of HaeIII (10 U μL−1, Fermentas Inc) in separate reactions. The volume was adjusted to 30 μL with nuclease-free water (Sigma-Aldrich) and incubated at 37°C overnight. Digestions were inactivated for 15 min at 85 °C and purified by ethanol precipitation (Stres et al., 2008, Likar et al., 2009). Separation, detection and basic GeneScan analysis of fluorescently labeled T-RFs were performed on an automated ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems Inc.). Purified digested products were mixed with 0.5 μL internal-lane DNA standard (Genescan 500 ROX, Applied Biosystems Inc.) and 10 μL deionized formamide. Before analysis, DNA samples were denatured for 2 min at 95 °C and immediately placed on ice. T-RFLP patterns with total fluorescence exceeding the limit of 10.000 units were analyzed and peaks representing 0.5% or less of the total community signal were excluded from further analyses in BioNumerics software (AppliedMaths, Sint-Martens-Latem, Belgium). Pearson's correlation coefficient with UPGMA clustering (for unweighted pair-group method with arithmetic averages) was used to derive general similarity of community profiles and the significance of clusters was determined using cluster-cutoff tool in BioNumerics (Stres et al., 2008, Likar et al., 2009; Novak et al., 2011).

2.2 Preparation of archaeal and bacterial 16S rRNA gene clone libraries and sequence analysis

Clone libraries (bacterial and archaeal) were constructed as was described previously (Kraigher et al., 2008). DNA preparations were used as a template for PCR amplifications of 16S rRNA genes PCR was performed with following primers; 109f (5' - CAN GCT CAG TAA CRC GYR-3') and 915r (5'- GTG CTC CCC CGC CAA

TTC CT-3') were used to amplify the 16S rRNA gene of methanogenic archaeal community (Watanabe et al., 2004) and 27f (6-FAM 5' - AGA GTT TGA TCC TGG CTC AG-3'), 926r (5'-CCG TCA ATT CCT TTR AGT TT-3'), were used to amplify the 16S rRNA gene of bacterial community, with the thermo cycling conditions used for T-RFLP. Products from three independent PCR amplifications were sliced from 1.5 % agarose gel and purified with Qiaquick PCR Gel Extraction Kit (QIAGEN, Stanford, CA, USA), and cloned into pGEM̀-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's instructions, after which competent high efficiency JM109 E. coli cells (Promega, Madison, WI, USA) were transformed and plated on LB (Luria-Bertani) plates supplied with ampicillin, IPTG and X-gal. White colonies were screened for inserts of the expected size (about 900 bp) using the vector primers SP6 and T7 (Promega, Madison, WI, USA). From each clone library, 288 clones were selected for sequencing by Macrogen Inc. (Seoul, Korea).

CodonCodeAligner program (http://www.codoncode.com/aligner/index.htm) was used to remove the remaining vector sequences left after sequencing and to define bacterial or archaeal 16S rRNA sequence stretches of sufficient quality. The refined sequence datasets were analyzed using Libcompare utility of Ribosomal Database Project (Wang et al., 2007; Cole et al., 2009) to identify which microbial groups within clone libraries were significantly different between the two microbial biomasses. Sequences were also imported to program ARB (Ludwik et al., 2004) for detailed phylogenetic analyses and construction of phylogenetic trees.

The phylogenetic analysis was performed using the ARB (version 5.2; Ludwig et al., 2004) and 2011 ARB-Silva database of reference dataset of bacterial 16S rRNA gene sequences (release 104; Pruesse et al., 2007). Sequences were automatically aligned using the Fast Aligner and the alignments were manually checked and corrected when necessary. Aligned, partial 16S rRNA gene sequences were inserted into the reference tree using a parsimony tool and positional variability by parsimony filter for bacteria, generated with complete sequence data, within ARB – Silva 109 database. Phylogenetic trees were edited using XFig software (version 3.2.5b).

2.3 Analysis of methanogenic microbial community by microarrays

In the 3rd and 5th experiment methanogenic microbial community was analyzed by microarrays essentially as was described previously (Frank-Whittle et al., 2009). Annex 3 lists the sequence, specificity, melting temperature and secondary structure details of all 101 probes.

Genomic DNA was extracted from the sludge using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California) according to the instructions of the manufacturer. Triplicate DNA extractions were conducted from the well-mixed bulked sludge sample. Extracted DNA was subjected to electrophoresis in a 1% agarose gel in 1× TAE buffer, and DNA concentration was determined by fluorescence using a PicoGreen® dsDNA quantitation kit (Molecular Probes Inc., Oregon, USA) and an fmax Fluorescence Microplate Reader (Molecular Devices, CA, USA), as described by the manufacturer.

2.4 Preparation of fluorescently labeled target DNA by PCR

The 16S rRNA genes of methanogens in the sludge were amplified by PCR using the 109F and 934R primers (Grosskopf et al., 1998). The 109F primer was Cy5- labeled and the 934R primer was labeled with a $PO4^-$ group at the 5′ end (Buchholz-Cleven et al., 1997). PCR amplifications were performed in a ThermoHybaid PCR Express thermalcycler in 50 μL volumes, with each standard reaction mix containing a final concentration of 1× reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl pH 8.8, 1.5 mM $MgCl_2$, 0.01% Tween 20) (GeneCraft, Munster, Germany), 200 μM each dNTP, 0.8 μM of forward primer, 0.2 μM of reverse primer, 10 mM TMAC (tetramethylammonium chloride), 0.1 μg/μL bovine serum albumin, 1.25 U BioThermTM DNA polymerase (GeneCraft, Munster, Germany) and sterile water. Between 0.5 and 2 μL of extracted biomass DNA (diluted 1/10 or 1/20) was applied directly to the PCR reaction mix. Positive and negative controls were included in each run. A positive control consisted of preanalyzed methanogenic archaea 16S rRNA genes. In negative control, sterile MQ was used instead of DNA in order to observe background fluorescence. Thermal cycling was performed according to a

modified protocol of Peplies et al. (2003). After an initial denaturation at 95 °C for 5 min, amplification reactions were subjected to 1 min at 80 °C, 1 min at 55 °C and 2 min at 72 °C. Thermal cycling then proceeded with 33 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. Temperature cycling was followed by 1 min at 95 °C, 1 min at 55 °C and a final extension at 72 °C for 10 min.

PCR products (three per DNA extract) were pooled and purified with the GenEluteTM PCR Clean-Up Kit (Sigma, Missouri, USA), and for the subsequent preparation of fluorescently labeled single-stranded DNA targets, the phosphorylated DNA strand was removed. Thousand ng of a purified PCR product was digested using 18U Lambda exonuclease (Epicentre, Madison, Wisconsin, USA) in 1× Lambda exonuclease buffer, at 37 °C for 3 h.

2.5 Hybridization

Single-stranded Cy5-labeled PCR product was vacuum dried and resuspended in 18 μL of hybridization buffer consisting of 5× SSC, 1% blocking reagent (Roche, Mannheim, Germany), 0.02% SDS, 0.1% n- laurylsarcosine, and 5% formamide (Loy et al., 2002). As a hybridization control, 1 μL of a 100 nM Cy5-labeled control oligonucleotide was added to each tube, and the mixture was denatured for 10 min at 95 °C, before being placed on ice. The solution was transferred onto a pre-chilled microarray (on ice) and covered with a glass cover slip to guarantee a uniform moistening of the array surface. Arrays were placed in a moistened chamber in a microarray oven (Hybex, CA, USA) and hybridization was conducted at at 55 °C. After hybridization, slides were washed immediately at room temperature, each for 3 min in buffer 1 (1× SSC, 0.2% SDS), followed by buffer 2 (0.1× SSC, 0.2% SDS) and buffer 3 (0.05× SSC). After a brief immersion in distilled water, the arrays were dried. Three arrays were hybridized using amplified, purified and digested DNA from the triplicate DNA extractions of the sludge.

2.6 Scanning of arrays and image analysis

Microarray slides were scanned with a ScanArray Gx microarray scanner (Perkin Elmer, MA, USA). Scan power was set to 90% and PMT gain to 500 at 633 nm for scanning. Fluorescent images were analyzed using the ScanArray Gx software (Perkin Elmer, MA, USA) by superimposing a grid of circles onto the image to designate each fluorescent spot to be quantified. The median foreground and background signals for all spots were determined. The signal to noise ratio (SNR) for all spots was calculated according to the following calculation, as described by Loy et al. (2002):

$$SNR = [I_p - (I_{np} - I_{bnp})] / I_{bp}$$

Where I_p is median intensity of fluorescence of the probe, I_{np} is the median intensity of fluorescence of the non-binding control probe, I_{bnp} is the median intensity of fluorescence of the background area around the non-binding control probe, and I_{bp} is the median intensity of fluorescence of the background area around the probe. Signals were assumed to be positive if an SNR value of ≥ 2 was obtained (Loy et al., 2002). The resulting datasets were imported into CANOCO software 4.5 (Microcomputer Power; Ithaca, New York, USA) (Braak and Šmilauer, 2002) for visualization of their similarities and identification of the key probes explaining the major part of data variability (Stres et al., 2010).

3 RESULTS AND DISCUSSION

3.1 T-RFLP fingerprinting of bacterial and archaeal communities from CSTR and UASB reactors

Bacterial microbial communities from CSTR showed marked differences upon incubation in BMP test (77% dissimilarity) after three applications of 2 mg /L cyanide as can be seen from Fig. 54. The mere inclusion in the BMP assay caused large changes in the structure of microbial community in both; the negative control (only biomass without addition of any substrate) and positive control that received glucose as a substrate. These two final states formed a set distinct from the initial state. The third set represented the final state, in which glucose and cyanide were added as substrates. Rearrangements of microbial community structure in glucose and cyanide amended samples were larger than those at the expense of glucose and significantly different from the changes that have taken place only at the expense incubation.

On the other hand, the addition of glucose as substrate had similar effect as addition of glucose and cyanide together at the level of archaea. This suggests that the pure effect of cyanide on the structure of archaeal microbial community in the time span of 60 days was small in comparison to the effects of glucose. Initial state and final state without substrate next to the final state with glucose and the final state of glucose and cyanide together represent two distinct clusters at 25% dissimilarity, suggesting substantial rearrangements in archaeal community response to substrate addition (Fig. 54).

The effect of added cyanide on the lag-phase in biogas production (Chapter 3) and the structuring effects on bacterial microbial communities (Fig. 54 A) suggest that the structure of bacterial microbial communities was significantly more responsive to changes in the environment than methanogenic archaeal microbial communities from CSTR.

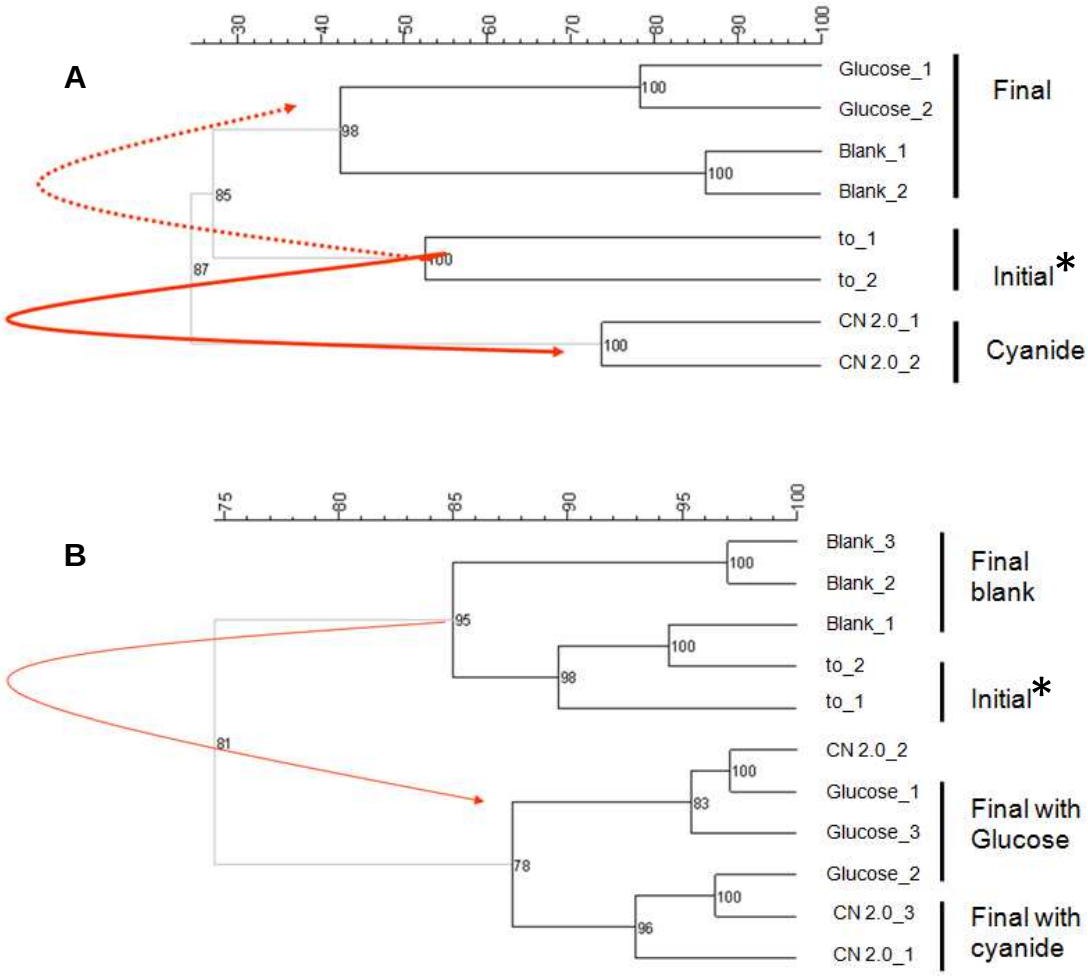

Fig. 54**:** *Pearson correlation dendrogram of T-RFLP fingerprints of methanogenic microbial communities originating from 3rd experiment (Chapter 3). The impact of free cyanide on the structure of bacterial (A) and archaeal (B) microbial community present in CSTR biomass from WWTP is shown (t0 - initial state, Glucose – positive control at the end of experiment t63, Blank – negative control at the end of experiment t63, CN 2.0. glucose + 2mg/L cyanide at the end of experiment t63). Horizontal gray lines delineate relevant clusters separated by the cluster-cut off method and similarity values (cophenetic correlation) are shown as numbers at each branch. The samples subjected to detailed analysis through cloning and sequencing are marked with an asterix.*

Bacterial microbial communities from UASB reactor showed differences upon incubation in BMP test in presence of 5 and 8.5 mg /L cyanide (Fig. 55 A). Changes were of smaller magnitude (55% dissimilarity) as those in CSTR amended with 2 mg /L of cyanide (77% dissimilarity) (Fig. 54 A). The mere inclusion in the BMP assay caused large changes in the structure of microbial communities with some indication

that increased cyanide concentrations resulted in distinct bacterial populations (three almost separate clusters at 25% dissimilarity). However, this could not be completely discerned. The T-RFLP profiles from positive control that received brewery wastewater as a substrate somehow branched with 5 mg /L cyanide samples.

On the other hand, the addition of brewery wastewater, cyanide in the two concentrations resulted in no measurable effect at level on archaeal community present in UASB reactor (12% dissimilarity) in comparison to CSTR biomass (Fig. 55 B). This suggests that the pure effect of cyanide on the structure of archaeal microbial community in the time span of this experiment was rather small and indiscernible from the background random signal (Fig. 55).

In conclusion, the lack of inhibition to added cyanide in biogas production (Chapter 3) and the smaller rearrangements in bacterial microbial communities suggest that the structure of UASB bacterial microbial community needed lesser rearrangements to maintain functionality than CSTR microbial community. It is also evident that CSTR microbial community bacterial and archaeal, experienced greater rearrangements both at the level of bacterial and archaeal microbial communities than UASB microbial community. The same pattern, that bacterial part of microbial community was more responsive to environmental changes was evident in CSTR and UASB samples, albeit at greater rearrangements present in CSTR biomass than in UASB.

However, the completely different functional performance of these two reactors could be traced to the differences in bacterial and archaeal microbial communities at the level of its constituents.

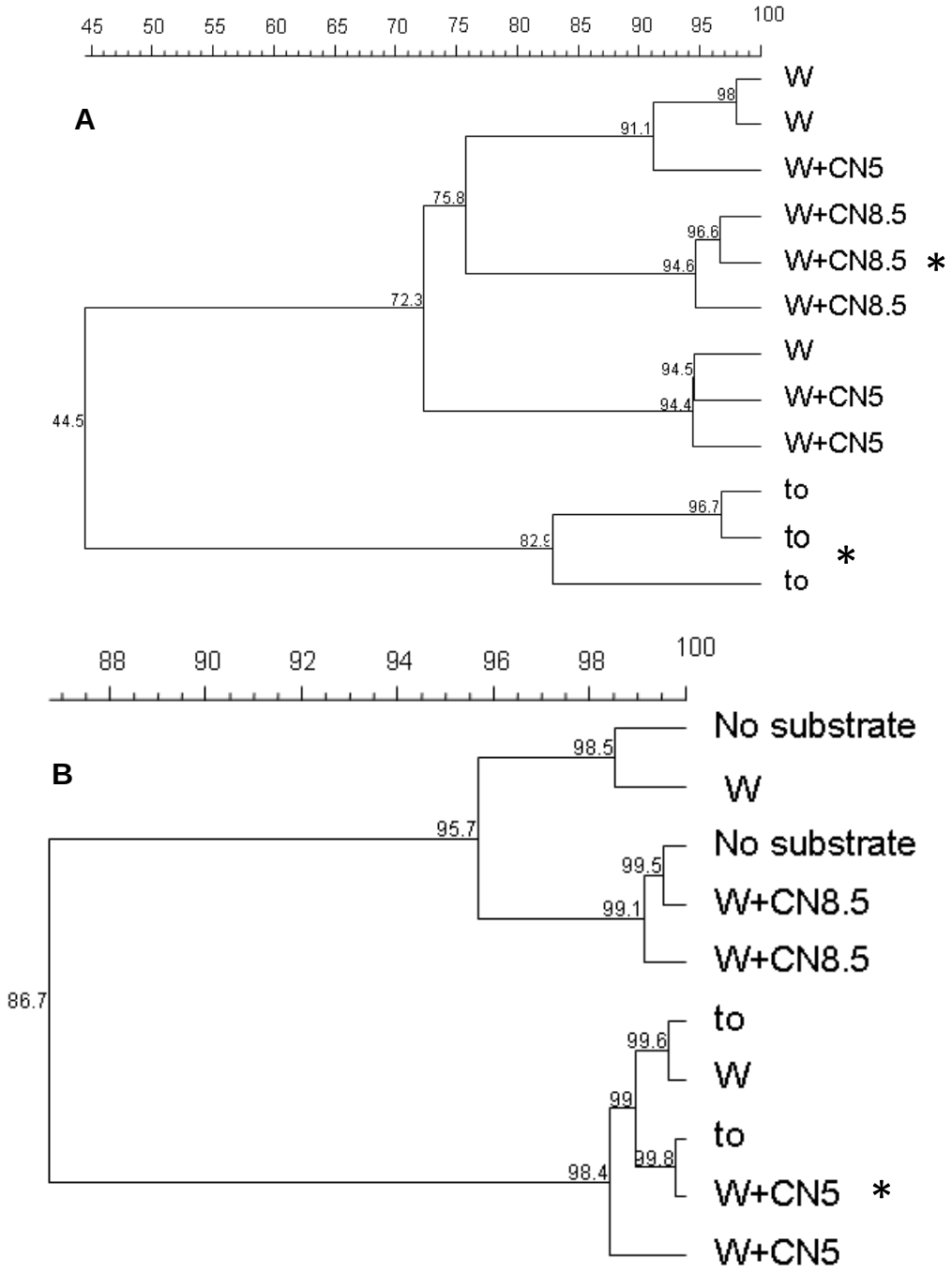

Fig. 55: *Pearson correlation dendrogram of T-RFLP fingerprints of methanogenic microbial communities originating from 5th experiment (Chapter 3). The impact of free cyanide on the structure of bacteria (A) and archaea (B) microbial community present in UASB biomass from an industrial reactor is shown (to – initial state; W –positive control fed with brewery waste water at the end of experiment t60; W+CN5 – brewery waste water amended with 5 mg /L cyanide at the end of experiment t60; W+CN8.5 - brewery waste water amended with 8.5 mg /L cyanide at the end of experiment t60). Horizontal gray lines delineate relevant clusters separated by the cluster-cut off method and similarity values (cophenetic correlation) are shown as numbers at each branch. The samples subjected to detailed analysis through cloning and sequencing are marked with an asterix.*

3.2 Detailed analyses of CSTR and UASB microbial communities through cloning and sequencing

After cloning and sequencing and initial data trimming and organization, the composition of the following datasets was compared: (i) initial bacterial CSTR vs. UASB composition; (ii) initial archaeal CSTR vs. UASB composition and (iii) developed control bacterial UASB vs. cyanide amended UASB community composition. The following differences could be identified using Libcompare utility:

(i) initial bacterial CSTR microbial community vs. initial UASB microbial community composition

The major differences between the two bacterial microbial communities at the level of bacterial microbial communities could be observed in relative abundance of three phyla: *Synergistetes, Spirochaetes* and *Cloroflexi* but not in the within phyla or lower taxa diversity (Table 15).

The phylum *"Synergistetes"* (Jumas-Bilak et al., 2009) includes a wide variety of genera, including *Aminobacterium, Aminomonas, Aminiphilus, Anaerobaculum, Cloacibacillus, Dethiosulfovibrio, Jonquetella, Pyramidobacter, Synergistes, Thermanaerovibrio*, and *Thermovirga*. "*Synergistetes*" are widely distributed in the environment, form part of the normal gut microbiota of animals (Godon et al., 2005), and have also been isolated from a variety of sites in humans, including the oral cavity (Vartoukian et al., 2009). There were also reports of presence in anaerobic digesters (Krakat et al., 2011, Riviere et al., 2009), but they were not clearly linked to process parameters. Vartoukian et al. (2007) reported, that *Synergistetes* could use amino acids and in turn provide short-chain fatty acids and sulphates for terminal degraders such as are methanogenic arcahea and sulphate-reducing bacteria.

Spirochaetes are widespread in aquatic environments and in animals. Shin et al. (2010) reported that bacterial community from two stage anaerobic reactor treating food-waste recycling wastewaters contains *Spirochaetes*. Seeded biomass originated from anaerobic digester of full-scale wastewater treatment plant. Krakat et al. (2011) also reported the presence of *Spirochaetes* in an anaerobic fermentor. The fermentor

was inoculated with a mixture of swine manure, sewage sludge and a hot-rot compost suspension. *Spirochaetes* were also identified as predominant in a mesophilic anaerobic community degrading oleate and palmitate in another study of (Shigematsu et al., 2006). They suggested that predominant phyla of *Spirochaetes* in the chemostat might play important roles in LCFA degradation. The seed sludge was a mesophilic-digested sludge from the sewage treatment plant. In all cases we can observe that biomass originated from sewage sludge. Since our microbial biomass with *Spirochaetes* was from granulated sludge, we can assume that was contaminated with municipal wastewaters. On the contrary, there was no sign of phylum *Spirochaetes* in suspended sludge from the CSTR reactor.

In the samples from CSTR reactor, *Chloroflexi* was found as prevalent group. *Chloroflexi* is abundant member of anaerobic digester treating various of substrates (Rivere et al., 2009, Paltasi et al., 2011, Krakat et al, 2010, 2011 Sekiguchi, 2006) *Chloroflexi*, together with *Clostridia* are known as potential hydrogen producers or users. Members of the phylum *Chloroflexi* (using mainly different carbohydrates and amino acids as substrate for growth) have been found previously in a wide range of anaerobic environments. This included anaerobic bioreactors operating under both mesophilic and thermophilic conditions, treating different kinds of waste (Sekiguchi et al., 1998, Yamada et al., 2005). Some representatives of this phylum are photolithoautotrophic and can use H_2/CO_2 as energy and carbon source (Zarzycki et al., 2009). Group of *Chloroflexi* was described as a "semi-syntrophic" partner of methanogens requiring inter-species hydrogen transfer for efficient growth (Sekiguchi, 2006). This indicates that *Chloroflexi* and abundant hydrogenotrophic *Euryarchaeota* (Krakat et al., 2010) compete for hydrogen. The phylum *Chloroflexi* is divided into two orders: *Chloroflexales*, the obligate or facultative phototrophs, and *Herpetosiphonales*, the aerobic heterotrophs (Garrity, 2001). Non-photosynthetic *Chloroflexi* have been frequently reported in reactors decomposing solid wastes (Rincon et al., 2006, Grotenhuis et al., 1991, McMahon et al., 2001).

***Table 15**: Differences in clone numbers/ clone libraries/ bacterial microbial community/ from biomass from CSTR and UASB reactor. Groups with significant differences are marked with* *

Biomass from CSTR	Phylum	Biomass from UASB
%		%
0.4	Candidate division OP10	0.0
3.6	*Synergistetes**	33.3
12.0	*Proteobacteria*	10.3
1.3	*Planctomycetes*	0.9
0.4	*Caldiserica*	0.0
0.9	*Thermotogae*	0.9
11.6	*Firmicutes*	18.8
4.9	*Bacteroidetes*	1.7
4.9	*Actinobacteria*	0.0
0.0	*Spirochaetes**	13.7
21.3	*Chloroflexi**	6.8
38.7	Unclassified Bacteria	13.7

(ii) Initial archaeal CSTR vs. initial UASB microbial community composition

Both archaeal communities were shown to be highly monophyletic and were composed of phylum *Euryarchaeota* (Table 16). The vast majority of CSTR archaeal microbial community (97.9%) belonged to class *Methanomicrobia* whereas UASB archaeal microbial community was represented by class *Methanobacteria* (81.8%). Within 97.9% of class *Methanomicrobia* in CSTR suspended sludge there was 95% of genus *Methanosaeta spp*., which are Gram-negative cells of about 0.8-3.3 µm size with typical bamboo-like rod shape and flat ends. Conglomerations mostly of many hundreds of single cells are surrounded by a layer of mucilage, which consists of fibrous glycol proteins. *Methanosaeta* takes only acetate as energy source, which is degraded to methane and CO_2. *Methanosaeta* has a high affinity and a very low threshold concentration to usual substrates. Therefore it can be found especially at places with low acetate concentrations. *Methanosaeta* is acetotrophic methanogen that play a major role in the metabolism of carbon in anoxic ecosystems in which electron acceptors such as nitrate, sulfate, and ferric iron are not available (Zinder, 1993). *Methanosaeta* as acetotrophic methanogen compete with other acetotrophic methanogens (*Methanosarcina*) for acetate. *Methanosarcina* are able to grow with

other methanogenic substrates, such as hydrogen, formate, and methanol, in addition to acetate, and display higher growth rates, lower affinities for acetate and higher thresholds for acetate utilization than *Methanosaeta*, which grow only with acetate (Jetten et al., 1992). These differences in growth with acetate mean that *Methanosaeta* dominate at low steady-state acetate concentrations, while *Methanosarcina* dominate when acetate concentrations are higher. When acetate is the substrate for methanogenesis, it is first activated to acetyl Co-A, which interacts with carbon monoxide dehydrogenase of acetyl Co-A pathway (Fig. 2 – introduction.)

The other 3 % of archaea belonged to the genus *Methanospirillum spp*., which has spirillum shape. All species take H_2/CO_2, formate as substrate for methanogenesis.
From the results describing microbial community structure in CSTR biomass we can conclude that acetoclastic methanogenesis is the dominant and prevailing metabolic pathway leading to methane formation in CSTR microbial biomass.

***Table 16**: differences between the initial CSTR and UASB archaeal communities. Groups with significant differences are marked with **

Biomass from CSTR	class	Biomass from UASB
%		%
1.1	*Methanobacteria**	81.8
97.9	*Methanomicrobia**	18.2
1.1	*Unclassified Euryarchaeota*	0.0.

Within 81.8 % of class *Methanobacteria* in UASB granulated sludge, 79 % belonged to genus *Methanobacterium*. They live in mesophilic and thermophilic conditions and some species prefer an alkaline environment. All species take H_2/CO_2, formate as substrate for methanogenesis. Based on the results from microbial community structure we can conclude that in UASB granular biomass, probably hydrogenotrophic methane formation prevails. The reduction of CO_2 with H_2 is the major pathway of methanogenesis in this case. About 20% of the archaeal community was composed of acetoclastic methanogens, similar to CSTR reactor.

***Table 17**: Differences in archaeal lineages represented in archaeal clone libraries form CSTR and UASB reactor.*

	Name	Library 1 CSTR	Library 2 UASB	Significance
Class	*Methanobacteria*	2	63	1.23E-31
Family	*Methanobacteriaceae*	2	63	1.23E-31
Order	*Methanobacteriales*	2	63	1.23E-31
Genus	*Methanobacterium*	2	63	1.23E-31
Class	*Methanomicrobia*	183	14	2.23E-14
Genus	*Methanosaeta*	177	12	6E-14
Family	*Methanosaetaceae*	177	12	6E-14
Order	*Methanosarcinales*	178	12	6E-14
	Unclassified *Methanomicrobia*	0	1	NA
	Unclassified *Methanomicrobiales*	0	1	NA
Family	*Methanospirillaceae*	5	0	2.53E-1
Genus	*Methanospirillium*	5	0	2.53E-1
Order	*Methanomicrobiales*	5	1	6.95E-1
	Unclassified *Euryarchaeota*	2	0	NA
	Unclassified *Methanosarcinales*	1	0	NA
Phylum	*Euryarchaeota*	187	77	1.03EO
Domain	*Archaea*	187	77	1.03EO
norank	*Root*	187	77	1.03EO

Gijzen et al. (2000) put forward the hypothesis that hydrogenotrophic methanogens are less sensitive to cyanide compared to acetoclastic methanogens. Based on T-RFLP analysis of our samples this could be true (Fig. 54, Fig. 55). In CSTR biomass we observed less effect of cyanide on archaeal microbial community in UASB reactor (12% dissimilarity) in comparison to CSTR biomass (25% dissimilarity). It was also evident that CSTR biomass experienced greater rearrangements both in bacterial and archaeal microbial community, than UASB microbial biomass. Since the cloning and sequencing revealed that in archaeal community in CSTR biomass 97.9% of phylum *Euryarchaeota* belonged to class *Methanomicrobia* (Genus *Methanosaeta*), which are acetoclastic methanogens, we can conclude that acetoclastic methanogens are actually more sensitive to cyanide in comparison to

hydrogenotrophic methanogens (*Methanobacteria*), which were predominantly present in UASB biomass. Gijzen et al. (2000) also suggested that higher cyanide inhibition in the case of acetoclastic metabolism may be explained by the sensitivity of carbon monoxide dehydrogenase enzyme as was suggested by Eikmans et al. (1985). This constitutes the first data providing grounds for the acceptance of the described hypotheses in the literature. Enzymes and cofactors involved in hydrogenotrophic pathway of methane production are apparently less sensitive to cyanide (Gijzen et al., 2000).

(iii) developed control bacterial UASB vs. cyanide amended UASB community composition

After 60 days of incubation bacterial microbial community was tested for final samples. We examined the structure of bacterial community in positive control and cyanide sample with initial concentration of 8.5 mg/L KCN. The differences between frequencies of bacterial phylogenetic lineages in both samples are shown in Table 13. The main difference was in the abundance of phylum *Firmicutes*.

***Table 18**: Differences in frequencies of bacterial phylogenetic lineages in positive control and cyanide sample at final state (60 days) For Firmicutesphylum, class and family are also shown. Groups with significant differences are marked with **

Positive control (%)	**Phylum**	Cyanide 8.5 mg/L KCN (%)
0.9	*Thermotogae*	0.0
0.9	*Planctomycetes*	0.0
10.3	*Proteobacteria*	1.2
6.8	*Chloroflexi*	1.2
13.7	*Spirochaetes*	5.9
33.3	*Synergistetes*	27.1
1.7	*Bacteroidetes*	3.5
19.7	*Firmicutes**	48.2
Class (within *Firmicutes*)		
3.4	*Clostridia*	2.4
3.4	*Erysipelotrichi*	0.0
12.0	*Bacilli**	45.9
0.9	*Unclassified firmicutes*	0.0
Family (within Firmicutes)		
0.9	*Paenibacillaceae*	8.2
11.1	*Bacillaceae**	36.5
0.0	*Unclassified Bacillales*	1.2
	Phylum	
12.8	Unclassified bacteria	12.9

In the cyanide sample *Firmicutes* was the most abundant group, representing 48.2 %, meanwhile in positive control sample *Firmicutes* represented 19.7 %. The prevalent group in control sample was *Synergistetes* with 33.3 % (in cyanide sample 27.1 %). Most of the clones affiliated within *Firmicutes* fell into the *Bacilli* cluster (45.9% of total clones in the library), meanwhile *Clostridia* cluster represented 2.4 % of total clones in library. Among the class *Bacilli*, detected groups were related to two families, namely *Bacillaceae* (36.5 %) and *Paenibacillaceae* (8.2%), and 1.2 % to unclassified *Bacillales*.

Yabu et al. (2010) reported, that dominant phylum in ammonia production was *Firmicutes* in two stage dry anaerobic digestion of garbage. This could indicate formation of ammonia through hydrolytic reactions after cyanide application (see. pp 48) and thus good conditions for grow of *Firmicutes*. This could be evidence of hydrolytic pathway at anaerobic degradation of cyanide in our system and confirmation of the thesis that anaerobic degradations of cyanide occur by abiotic/ biotic processes.

There are reports of presence of *Firmicutes* in rather unusual or difficulty degradable substrates for anaerobic digestions. Grey et al. (2010) reported that *Firmicutes* are the most commonly encountered bacterial taxon in petroleum reservoir fluids. For instance, members of this group have been identified as the dominant microbial constituents in anaerobic enrichment cultures degrading benzene (Kunapuli et al., 2007; Abu Laban et al., 2009) or propane (Kniemeyer et al., 2007). Wang et al. (2011) suggested that *Firmicutes*-like organisms thrive in oil-rich habitats, e.g., oil reservoirs. It seems that they can adapt to use oil components *in situ* as well as in methanogenic enrichments such as the one described in the study of Wang et al. (2011), in which the n-alkanes were used as the sole carbon source. In their experiments, after 274 days of incubation there was more methane production in n-alkane sample than in control samples. We observed rather similar pattern, since there was more cumulative biogas and methane in cyanide sample, than in positive control. This could indicate that *Firmicutes* are quite resistant for different pressures from environment and this could be the reason for their growth in the presence of cyanides. We cannot be sure that they use cyanide as carbon or nitrogen source due to the lack of evidence.

In the future more powerful techniques such as SIP (Radajewski et al., 2000) and NanoSIMS 50L (Musat et al., 2008) could be used to test whether the observed pattern of phylum *Firmicutes* being associated with cyanide use as nitrogen source is correct or not. The initial project proposal was submitted, reviewed and will take place in the future.

In order to verify the observed differences in microbial communities on the level of 16S rRNA gene sequences obtained by Classifier utility of Ribosomal Database Project II a separate independent analysis employing a different approach using ARB software (Ludwig et al., 2004) was conducted. As Classifier and ARB use different approaches (secondary structure filters and metrics for sequence affiliation to phylogenetic lineages) the outcome results were compared and the phylogenetic trees were reconstructed.

The comparison of major lineages from phylogenetic trees generated in ARB and those from Classifier hierarchy clustering tables revealed that the two methods gave highly comparable and congruent results.

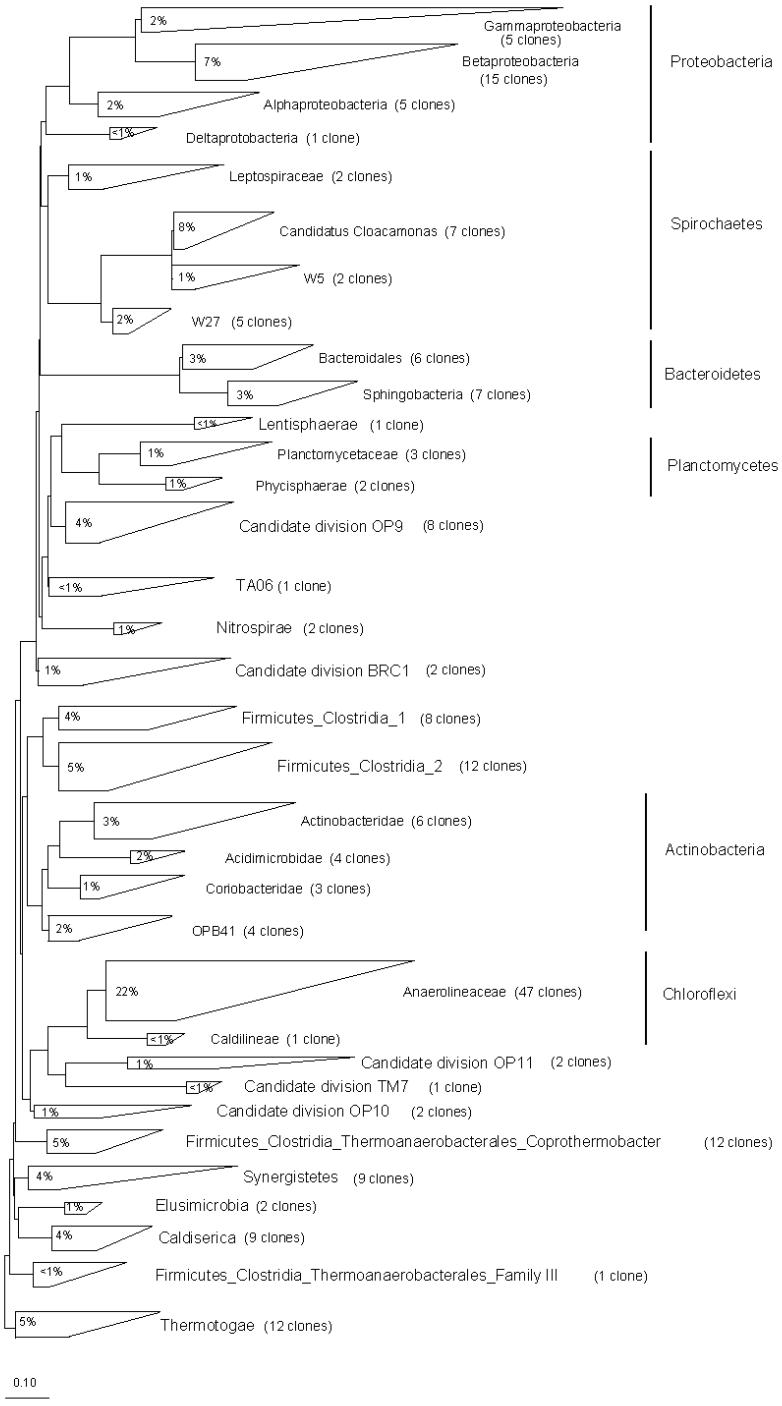

Fig. 56**:** *Phylogenetic affiliation of 16S rRNA gene sequences obtained from CSTR reactor. Phylogenetic tree is showing the position of representative clone sequences on the guide tree of the ARB-Silva database (Ref NR 99% 1200; release 104). The topology of the tree is based on maximum parsimony analysis of reference sequences. The share of clones that fell into each phylogenetic clade is indicated in the frame of defined clade and corresponding number of clones is shown in parenthesis. Scale bar estimates 10% sequence divergence.*

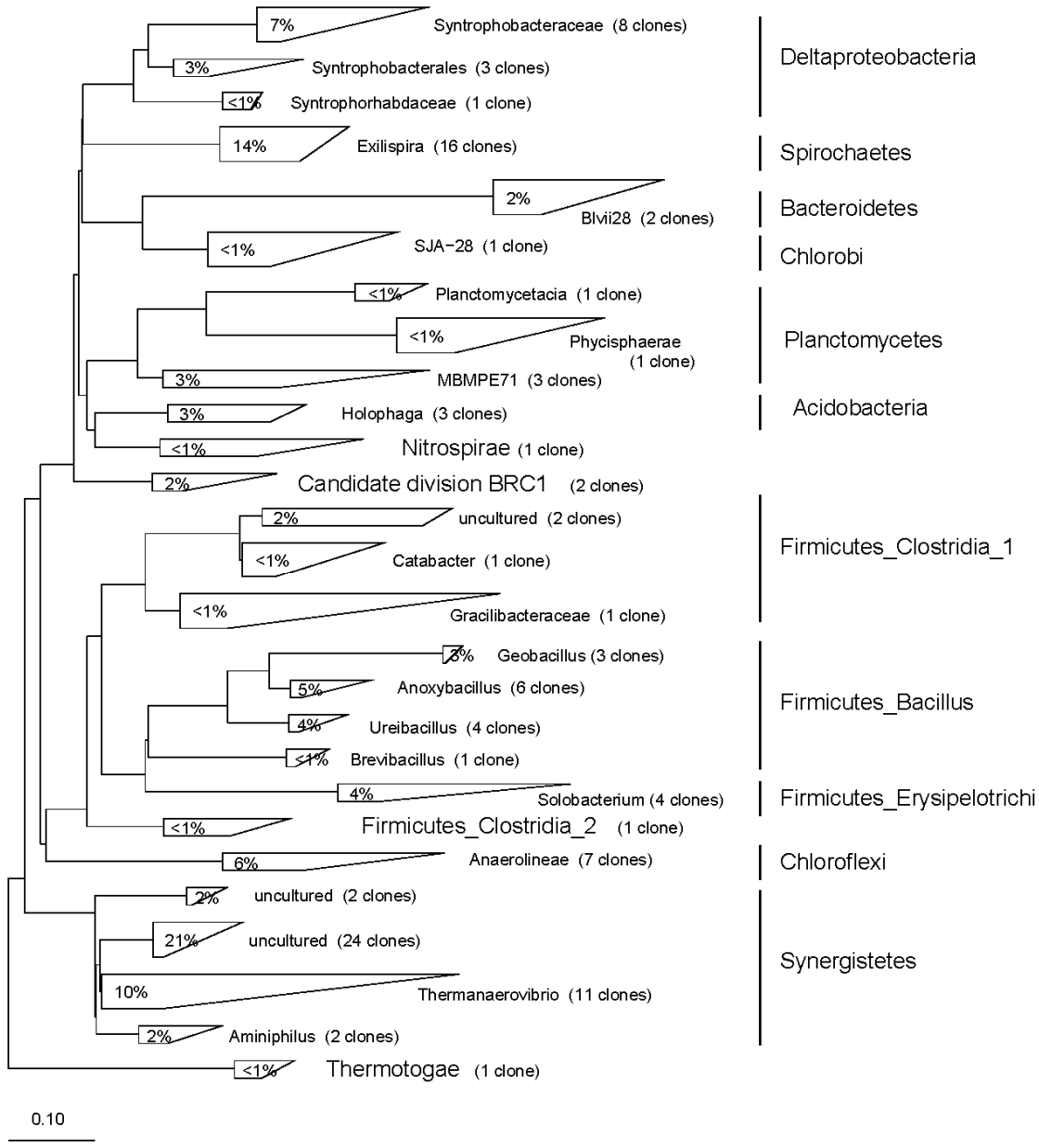

Fig. 57**:** *Phylogenetic affiliation of 16S rRNA gene sequences obtained from positive control (UASB reactor) at the end of incubation (t60). Phylogenetic tree is showing the position of representative clone sequences on the guide tree of the ARB-Silva database (Ref NR 99% 1200; release 104). The topology of the tree is based on maximum parsimony analysis of reference sequences. The share of clones that fell into each phylogenetic clade is indicated in the frame of defined clade and corresponding number of clones is shown in parenthesis. Scale bar estimates 10% sequence divergence.*

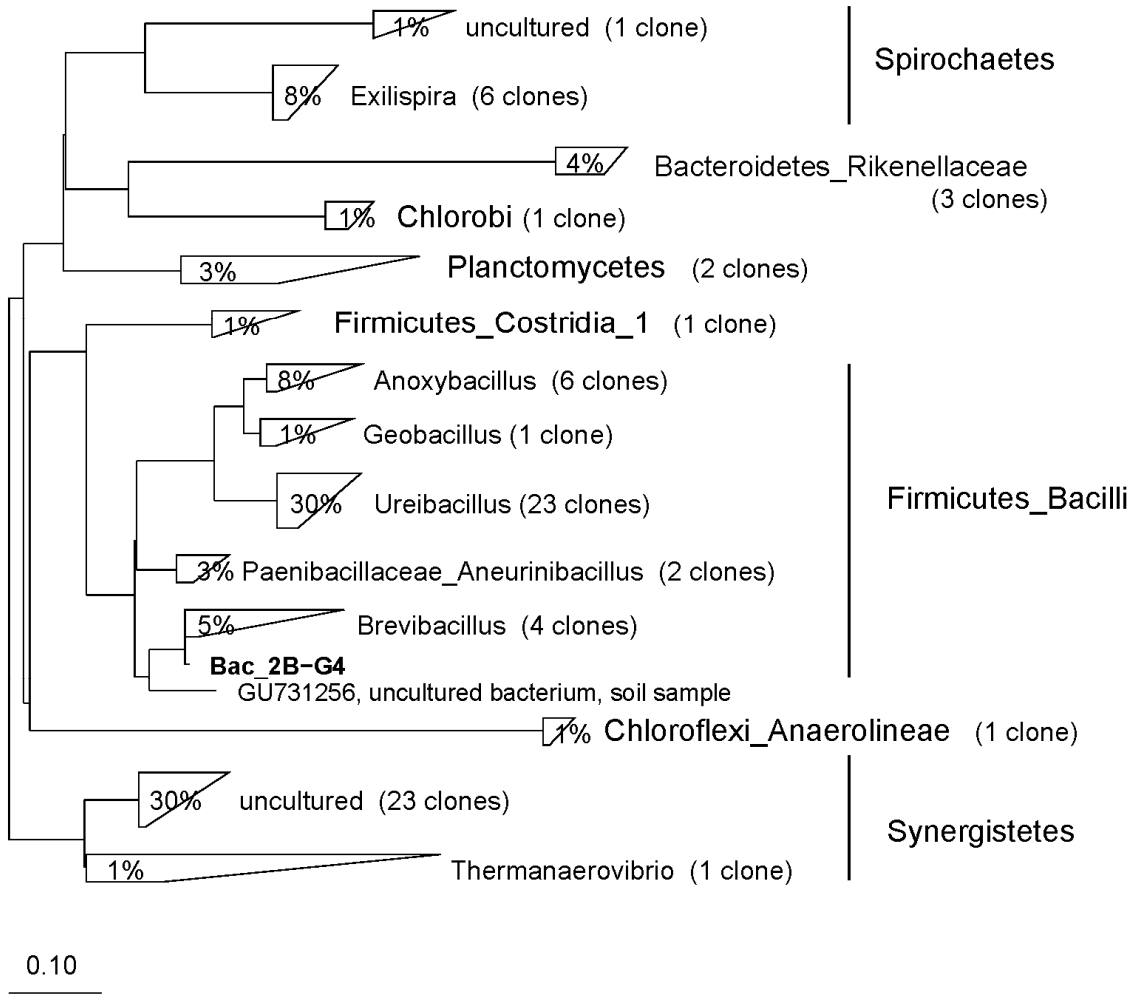

Fig. 58**:** *Phylogenetic affiliation of 16S rRNA gene sequences obtained from cyanide sample with initial concentration 8.5 mg/L KCN (UASB reactor) at the end of incubation (t60). Phylogenetic tree is showing the position of representative clone sequences on the guide tree of the ARB-Silva database (Ref NR 99% 1200; release 104). The topology of the tree is based on maximum parsimony analysis of reference sequences. The share of clones that fell into each phylogenetic clade is indicated in the frame of defined clade and corresponding number of clones is shown in parenthesis. Scale bar estimates 10% sequence divergence.*

3.3 Microarray analyses of archaeal microbial communities

Microarrays produce semi quantitative data due to the use of PCR-amplified templates during fluorescent labeling that may over represent the dominant groups of microbes. In addition, the inherent differences in hybridization efficiencies among various oligonucleotide probes may further affect the signal. However, recently,

highly significant correlations have been determined for the signals obtained from Anaerochip (Whittle et al., 2009) and quantitative PCR (Goberna et al., 2010). Thus, at least in terms of comparison of the dynamics of the dominant taxa in the unknown samples, Anaerochip may provide valuable information and sufficient high through put.

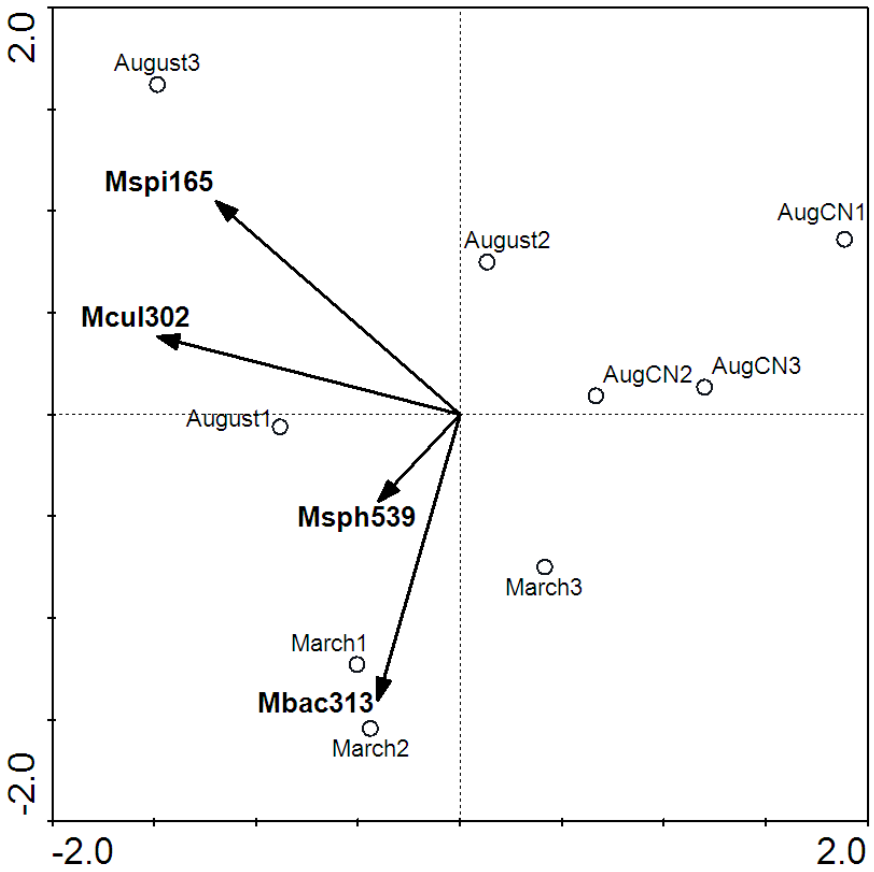

Fig. 59: *Canonical ordination of archaeal microbial community from CSTR biomass (open circles) from the initial state sampled from mother industrial scale reactor in March (March1-3) and August (August1-3) and from the developed communities in the 3rd experiment (Chapter 3; AugCN1, AugCN2, AugCN3). The arrows indicate the increase in the gradient of signal intensity of the most important microarray probes for methanogenic archaea associated with the distribution of the samples (Mspi165 - Methanospirillum; Mcul302-Methanoculleus; Mbac313 – Methanobacteriaceae; Msph539 – Methanosphaera). See text for further explanation.*

In case of CSTR data (March, August, August + cyanide) 21 probes out of 46 with signal intensity above 2 were significantly associated ($p<0.05$) with sample distribution. Only four probes out of 21 could be significantly linked with the sample

distribution after 499 permutation cycles ($p = 0.002$). The results show that CSTR samples collected in March from mother reactor could be differentiated from those collected in August and used in subsequent experiments for cyanide loading.

Albeit the differences could not be shown to be significant due to the data scatter, the results indicate rearrangements in the archaeal microbial community structure. In a time-line from March to August, a general decrease in *Methanobacteria* and *Methanosphaera* and enrichment in *Methanoculleous* and *Methanospirillum* in the March samples was observed. This suggests that natural oscillations in archaeal microbial communities occur on regular basis in mother CSTR, most probably due to differences in substrate identity, presence of inhibitory or recalcitrant substrates and the differences in their loading rates.

On the other hand, a decrease in relative abundance of all four most significantly associated probes can be observed in the August samples amended with cyanide (Fig 60) suggesting the start of drastic rearrangements in the archaeal communities towards more even communities as can be observed in anaerobic reactors under stress (Goberna et al., 2010). This is well in line with our previous observations (Chapter 4 – T-RFLP, cloning and sequencing) showing nearly significant changes in the archaeal microbial communities in response to cyanide presence in the time span of conducted experiments (21 days). With respect to the cyanide amended community the magnitude of rearrangements was in the same range as the seasonal variation, however, the unique direction shows a different stage in organization of microbial communities. Due to the high spatial variability the subtle changes that took place could not be observed in all of the microniches, an observation that is in line with our T-RFLP analyses of archaeal microbial communities.

A different set of probes was found to significantly explain the relationship between the UASB samples under analysis. In UASB data a set of 20 probes out of 46 with signal intensity above 2 were significantly associated ($p<0.05$) with sample distribution. Again only four probes out of 20 could be significantly linked with the sample distribution after 499 permutation cycles ($p < 0.01$). Although the rearrangements in the archaeal microbial communities were comparatively small and thus the changes could not be deemed significant as observed before in our T-RFLP

analyses (Chapter 4), the following observations on microbial rearrangements can be made: the results show that a general decrease in the abundance of *Methanobacterium* + *Methanothermobacter*. *Methanospirillum* and *Methanobrevibacter* could be observed as a result of incubation in BMP experiments and presence of 5 mg/L cyanide. This suggests that in the case of UASB biomass the incubation in BMP exerted effects of similar magnitude as the presence of 5 mg/L cyanide that did not prove to be inhibitory for methanogenesis, possibly canceling out each other to a certain degree as can be observed from the Fig. 60 where samples 4abc are above samples 3abc and out of the line drawn across samples 1abc-2abc-3abc and closer to 5abc samples than other samples suggesting that some rearrangements take place also due to the cyanide presence.

Further, members of *Methanosarcina* were the only archaeal group that were significantly associated with 8.5 mg/L cyanide, experimental variation in which 25 day lag phase before active methanogenesis was observed. The increase in their relative abundance in cyanide-amended samples is due to their tolerance for substances, which can be inhibitory to other methanogens, such as ammonia or high acetate concentrations. The higher volume-to-surface ratio of the coccoid *Methanosarcina* and its growth in clusters might explain increase tolerance to ammonia, cyanide, acetate or other inhibitory compounds as the presence and also dominance of members of *Methanosarcina* have been reported for many anaerobic environments including manure-digesting reactors (Goberna et al., 2010).

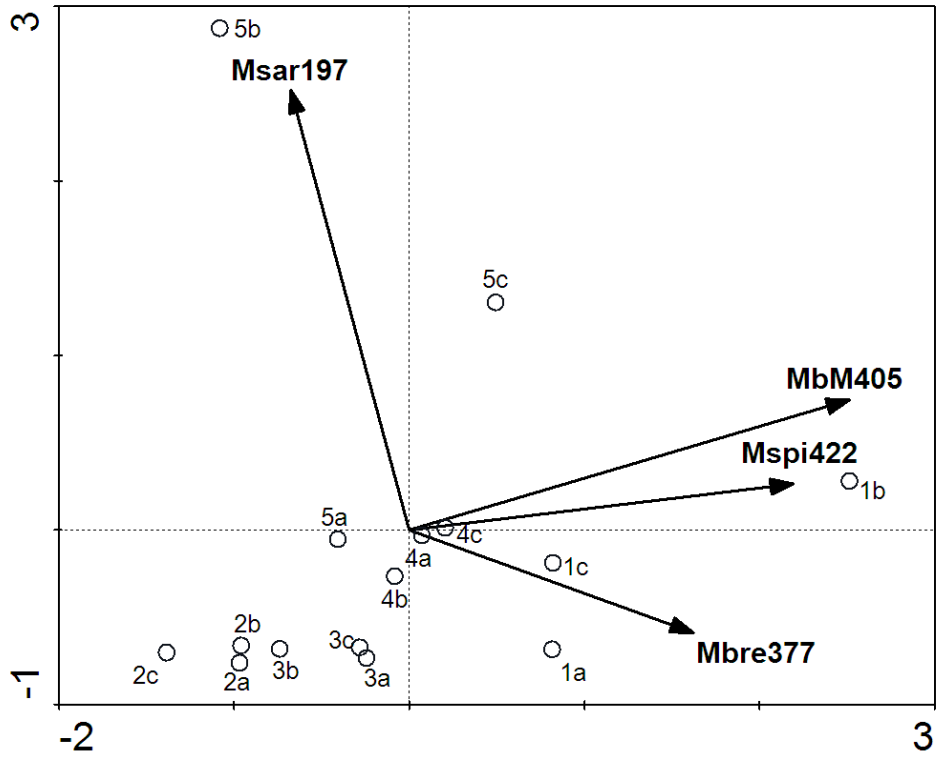

Fig. 60: *Canonical ordination of archeal microbial communities present in UASB biomass (open circles; 1abc – initial samples; 2abc – developed negative control; 3abc – developed positive control fed with brewery wastewater; 4abc – developed fed with brewery wastewater and 5 mg/L cyanide; 5abc – developed fed with brewery waste water and 8.5 mg /L cyanide). The arrows indicate the increase in the gradient of signal intensity of the most important microarray probes significantly associated with the distribution of samples (Msar197- Methanosarcina; MbM405-Methanobacterium and Methanothermobacter; Mspi422- Methanospirillum; Mbre377- Methanobrevibacter). See text for further explanation.*

The differences we described in the microbial community structure of archaea based on microarray data could not be verified to represent significant changes in microbial structure, a finding congruent with our observations made on the level of phylogenetic profiling using T-RFLP (Chapter 4). This shows that in the future analyses of microbial communities the sole choice of methodology does not fundamentally affect the experimental outcome and allows one to obtain congruent results by the linking of different methodologies: (i) Microarray vs. T-RFLP (this study) and (ii) Microarray and Q-PCR (Goberna et al., 2010).

Discrepancies between the methodological approaches however existed. Therefore we explored the methodological distinctions between clone libraries and arrays

further by identifying the few unidentical factors, the one could focus on in the future to additionally minimize data variability. As essentially the same primers and PCR conditions for the preparation of labeled and unlabeled PCR products for arrays and cloning and sequencing, respectively, were used next to the same PCR conditions, only the following differences could be identified: the number of cycles differed for 1 cycle, the nature of the two approaches: random cloning vs. diffusion constraints in arrays, and slightly different DNA extraction procedure (PowerSoil vs. Ultra CleanSoil, both MO-BIO, USA).

Nevertheless, in this work we independently showed using two methodological approaches that archaeal microbial communities did not significantly rearrange in the presence of cyanide as an additional carbon source. However, the use of Anaerochip provided the analyses of microbial communities through multiple probe approach showing the subtle rearrangements and initial trends in adjustments of archaeal microbial communities in response to functional challenges from the environment that need to be further explored. It is well known that functional degradation of microbial communities does not necessarily imply significant rearrangements at the level of microbial community structure. Recently, completely stable microbial community was shown to be functionally completely inhibited (Goberna et al., 2010). Thus, the subtle changes observed in our work provide grounds for significant rearrangements in microbial community structure at longer time scales than those used in experiments in this study.

CHAPTER 5

CHAPTER 5 – THESIS DISCUSSION AND CONCLUSIONS

In EU, including Slovenia, the number of biogas plants has increased during past years (7 new biogas plants in Slovenia between years 2007 - 2011). This can be attributed to governmental promotion for the construction of such plants and also to the higher rates for electricity derived from biogas. However, as more biogas facilities run on energy crops, such a corn silage and beet silage, non-governmental organizations (NGOs) have started to draw attention to the use of food for production of electricity. Their pressures on the government have brought or are at least beginning to bring changes in the Regulations governing this area. For example, for 2012 in Germany, which is the most developed country in the field of biogas production with over 5.000 installations across the country, there are no plans for new biogas plants, because all potential investors are waiting for a new regulation release specifying the use of substrates for biogas and related subsidies. No one knows exactly what the new regulation may bring to this field, but one can be certain: governments will not give subsidies for biogas production from so called energy crops, at least not at the current level of support. Therefore, one can expect more and more biogas plants that will be operated with more complicated or should we say with more persistent substrates in the future. These substrates include ligno-cellulose substrates (green trim, grass, crop residues…etc), substrates from different industry (industrial wastewaters containing different hardly degradable substances…etc). It is easy to make biogas with substrates with ideal C:N:P ratio, however it is far more complicated task to produce biogas from unknown substrates, which now are mostly deposited in landfill or neutralized via chemical procedures.

In the next few years, biogas operators are going to face new, unknown substrates because of the new law enforcement. In order to maintain the profitability of biogas production, reduce the intake of substrates from energy crops and increase the substrates that today mostly end on landfill or elsewhere. Such substrates have not been used before in biogas production and represent new and in most cases unknown substrates also for the microbial communities currently used. Disturbances of anaerobic digestion process, which are due to differences in the substrates or substrate concentration or loading rates can in worst-case lead to shutdown of the gas production. The consequent adaptation to the new conditions and subsequent

resumption of biogas production can last up to three or even more weeks resulting in higher (unsustainable) operating costs (Deublein et al., 2008). To ensure undisturbed and efficient production of methane and thus waste removal, it is necessarily to test any such unproven or unknown substrate by a rapid, reliable and economically affordable lab scale test before being used in the anaerobic degradation process on the full-scale level. Such a test can verify the biodegradability of a substrate with a precisely defined microbial biomass. On the basis of the test results one can evaluate biogas production and thus define appropriate parameters to reduce the risk of process failure. Numerous industries generate not only less degradable but even toxic substrates that can be used for biogas production. We tested one of those substrates in our research work-cyanide.

In the present Chapter 5 the overall findings presented throughout the thesis are comprehensively summarized following the quotation of initial aims (**in bold**) presented in the Introduction section of the thesis:

1. Aim: To establish the system for bio-monitoring process of anaerobic degradation by selecting and evaluating suitable experimental methods to be used throughout the study;

During our research work we established a system for bio-monitoring process of anaerobic degradation. We used the system (Oxitop®) (Voorthuizen et al., 2008, Fan et al., 2009 Bouzas et al., 2007) and optimized the system for our needs. We selected Oxitop® from among the many available setups, because it was economically favorable. We started our research work with the optimization of this system to meet our needs and comply with the ISO standard that defines anaerobic biodegradability (EN ISO 11734). We also customized and improved the existing program (AHAT OC, WTW, Germany) code for faster analysis of data recovered from the Oxitop® measuring heads. Data (pressure in hectopascals) was converted via general gas law into the volume of biogas, and they were graphically shown as the cumulative production of biogas. We added bio-monitoring of the anaerobic digestion process. This means that we added new parameter to the usual parameters (biogas composition, VFA, pH, NH_4-N, COD removal…etc), which are followed in an anaerobic digestion process. The novel parameter was tracking the microbial

community structure during anaerobic digestion to observe dynamics in microbial community and also structure-function relationship of methanogenic and also bacterial biomass in the event of substrate change.

At the beginning of our research work, we did not want to start with a complicated substrate, because our focus at this stage of the research was on optimizing the monitoring process. Thus, a microbial biomass from a well functioning UASB reactor was selected and incubated with a novel well degradable substrate (fresh yeast and hydrolyzed yeast). Using this setup, we followed all the usual parameters in biogas production with the Oxitop® monitoring system, and we also added new parameter; tracking of microbial community. Optimizing the available molecular techniques for our needs was time consuming. Extraction of DNA from our samples was not successful at the beginning of our research work although we used commercially available kit that was effective in extracting DNA from more complex samples like sediment or soil. To obtain reproducible DNA yields and subsamples from complex samples, we modified the protocols by removing the surplus liquids left after high-speed centrifugation by speed drying of biomass samples for the subsequent DNA extraction from such samples using DNA isolation kit for soil (MO-BIO, USA). After substantial optimization of PCR reaction composition, we were able to perform PCR from that DNA to get optimal results. Consequently, we believe we can use this same set up with any kind of biomass and substrate to evaluate biogas production and bacterial and archaeal community dynamics.

Therefore, with the use of molecular techniques in combination with already known methods for testing methane production, we established a bio-monitoring process, which can be further used for planning a stable anaerobic methanogenic process and also for routine monitoring of its stability. Thus, we established a system for bio-monitoring the degradation process effectiveness, community dynamics and structure-function relationship of methanogenic and also bacterial biomass in the event of substrate change.

2. Aim: To estimate the effects of a novel, less degradable substrate (brewery spent grain) on distinct bacterial and archaeal microbial communities;

The next step was evident. We wanted to estimate the effect of a novel and less easily degradable substrate on bacterial and archaeal microbial communities and to test our system in order to determine if our established methodology is able to track microbial community dynamics in our experimental systems, since there were no explicit reports of microbial community shifts in routine BMP assay, neither for bacteria or archaea. For the experiment we selected three different biomasses that were amended with a novel less degradable substrate – spent brewery grain. Biomasses originated from different types of anaerobic process; A - sewage sludge, B - urban organic waste and C - agricultural organic waste. The anaerobic digestion process was completed successfully providing the opportunity to check and evaluate possible microbial community dynamics. We used methods we developed for tracking microbial community (PCR, T-RFLP and statistical methods). The results presented in Chapter 2 revealed which selected biomass was most suitable for theoretical start up of new reactor treating brewery spent grain. It was not only that we were successful in tracking microbial community dynamics; we actually observed differences in archaeal microbial community during the incubation time in BMP assay. Based on the result, we were able to conclude that one of the biomasses used, the biomass from waste water treatment plant digester was most suitable for quick and reliable start up of a novel anaerobic reactor treating brewery spent grain as a novel substrate. To our knowledge, this may be one of the first studies, which provided methanogen community dynamics in all routine BMP assay variants (negative and positive controls plus experimentals) for three distinct biomasses and statistically compared the community shift profiles. It also extends well to ATA, as reanalysis of published data reversed the outcome of some of the previously published studies (Nyberg et al., 2008) and showed that the effects of different substances on microbial communities could be in fact detected.

With this second chapter of our research work we confirmed that the selected system is sufficiently robust as we were able to perform the analyses we established on four distinct biomasses (one - Chapter 1, three – Chapter 2).

The next step in our work was to apply the methodologies we developed to a more complicated substrate cyanide, that is known to exhibit multiple effects on the various levels of microbial processes and community dynamics, contributing much to the conflicting conclusions found in published literature on its biological degradation and potential for biogas production. Due to the complexity and chemical reactivity of cyanide, we anticipated that the environmental chemical composition could play a significant role in the observed conclusions presented by others in published literature on the degradation of the cyanide. Thus, two distinct biomasses, each with a distinctly complex environmental history, substrate diversity, quality and loading, were selected.

3. Aim: To determine the possibility of cyanide degradation in two distinct biomass samples in relation to previous findings (Gijzen et al., 2000, Fallon et al., 1991, Fedorak and Hrudey 1989) and to identify the key microbial constituents in the biomass samples involved in cyanide degradation

The published scientific literature does not hold many entries on anaerobic digestions of cyanide (Fedorak and Hrudey 1989; Gijzen et al., 2000; Fallon et al., 1991; Paixao et al., 2000; Annachhartre and Amornkaew et al., 2000; Chakarobrty et al., 2005). All authors reported successful anaerobic degradation of cyanide involved the capacity of the microbial communities present in anaerobic sludges to adapt to increasing concentrations of cyanide. However, there has been no report on the microbial community constituents responsible for cyanide degradation under anaerobic conditions under either laboratory or industrial conditions. We decided to fill this gap.

Well-known cyanide toxicity to all living beings is probably the main reason for its limited utilization in anaerobic degradation and biogas production. Hydrogen cyanide gas $HCN_{(g)}$, free cyanide in aqueous solution $HCN_{(aq)}$, and CN^- which can be converted to $HCN_{(aq)}$, and simple cyanide salts (e.g., NaCN, KCN) are the primary toxic forms of cyanide. The sites of action for cyanide toxicity are heart and brain. Cyanide is lethal in high doses (200 mg KCN in an adult human) but lower doses can damage sensitive brain cells (Gee, 1987). Experiment on laboratory mice revealed that dose of 5mg/kg KCN is reflected by a decrease in motor activity, but

consciousness is maintained and animals respond to prodding (Borowitz et al., 2001). However even low doses can damage sensitive brain cells (Pavlakovic et al., 1994; Fetcher et al., 2002). Although several cyanide antidotes are available, none of them can reverse the cyanide effects completely.

In our research work on cyanide, we used two distinctive microbial biomasses. One microbial biomass was from second stage (anaerobic CSTR) of WWTP; the second was granulated biomass from industrial UASB reactor. Experiments with CSTR biomass were not promising in terms of cyanide degradation because it contained metals among which was iron. In addition, the WWTP reported regular annual incidents of increased heavy metal inflow due to industrial activities upstream of the WWTP site.

In the 1st experiment with 1.5 mg/L of KCN, we observed perfect methanogenesis after a short lag phase (later found to be metal-cyanide complexation). The effects of the metal presence in the CSTR biomass were revealed after subsequent experiments with initial concentrations of 5.0 and 10.0 mg/L of KCN, which resulted in metal-cyanide complexes, probably $Fe(CN)^{4-}_6$ species as it was reported that this is prevailed form of ferrocyanide in anoxic conditions (Schecher et al., 1998). We found that direct application of 5.0 mg/L caused strong inhibition of methanogenesis, which is congruent with the results of Gijzen et al. (2000). Based on the results from experiments (1st three experiments) with CSTR biomass we concluded that added cyanide in CSTR biomass was transformed from free cyanide to metal-complexed cyanide and was thus not converted biologically. We also discovered, that beside lag phase in case of initial concentration 1.5 mg/L of KCN, we observed normal methanogenesis indistinguishable from positive control amended with glucose. Meanwhile in the case of initial concentration of 5.0 and 10.0 mg/L the process of methanogenesis completely inhibited for the entire duration of the experiment.

In the third experiment we used three applications of 2.0 mg/L of KCN during 63 days of incubation. After first two runs (each run lasted 21 days) we observed that a lag phase almost identical to that in experiment 1 preceded normal methanogenesis. However, in the last run after the third amendment of 2 mg/L cyanide the lag phase was significantly prolonged. Measurements of total cyanide revealed that free

cyanide was transformed to the metal cyanide complexes and thus accumulated to the concentration of 5.7 mg/L of total cyanide present in the environment. Nevertheless, the process of methanogenesis was more effective than after direct application of 5.0 mg/L of cyanide. Methane content in cumulative biogas at the last 3rd run was higher for 20.2 % than in the sample with direct 5 mg/L cyanide application. Based on that result we concluded, that metal-complexed cyanide was not biologically available for degradation, however it remained toxic in the present concentration for microbial communities in the microbial biomass. From the results it follows that microbiological community can adapt to the toxicity of metal-cyanide complexes to a certain degree (Gijzen et al., 2000), but nevertheless could still use cyanide as carbon or nitrogen source for growth. As in the first experiments with cyanide we did not observe significant cyanide removal, we concluded that complexation from free species to complex form with heavy metals is faster than reductive reactions at which, dissociated HCN is transformed to ammonia and methane. Dissociation of ferrocyanide under anaerobic conditions is to the best of our knowledge not possible due to conditions that need to be met (pH 1-2 at 125°C).

In the third experiment (adaptation experiment with 2 mg/L cyanide), we also explored microbial communities at bacterial and archaeal 16S rRNA levels by profiling with T-RFLP, microarray and cloning and sequencing. In terms of the dynamics of microbial communities in the 3rd experiment, the following observations were made: T-RFLP profiling of microbial community revealed the effect of added cyanide on the lag-phase in biogas production (Chapter 3) and the much larger structuring effects on bacterial microbial communities suggesting that the structure of bacterial microbial communities was significantly more responsive to changes in the environment than methanogenic archaeal microbial communities from CSTR.

Cloning and sequencing of biomass (from initial stage) revealed, that in the samples from CSTR reactor phylum *Chloroflexi* was found as prevalent bacterial group. The group *Chloroflexi* is abundant in anaerobic digester treating several substrates (Rivere et al., 2009, Paltasi et al., 2011, Krakat et al, 2010, 2011 Sekiguchi, 2006). In archaeal part of microbial community, class *Methanomicrobia* was found as the prevalent group (97.9 %) in the CSTR biomass. Most of the *Methanomicrobia* in this

biomass was assigned to *Methanosaeta*, which takes only acetate as energy source, which is degraded to methane and CO_2. From the results describing microbial community structure in CSTR biomass, we can conclude that acetoclastic methanogenesis is the dominant and prevailing metabolic pathway leading to methanogenesis in CSTR biomass.

In order to alleviate the effects of the heavy metals present in CSTR biomass, a pretreatment could be conducted in the future experiments composed of heavy metal complexation by direct reaction with hydrogen sulfide (H_2S) before biogas production. This would preclude the subsequent formation of cyanide-metal complexes for the majority of metals present in the biomass sludge. This is of course not a relevant environmental condition as metals and cyanide would be delivered in the same wastewater. From the experimental point of view that would enable the elucidation of the metabolic behavior of microbial communities and their genetic rearrangements in the absence of the major fraction of toxic cyanide-metal complexes. In addition, the use of identical substrate as carbon sources in both biomasses could also be attempted, however, rearrangements in microbial communities as a result of novel substrate for one of the biomasses or both would thus need to be taken into account.

In the second half of experiments we used granulated biomass from industrial UASB reactor. Since our experience with biomass that contained metals, we wanted to test biomass without noticeable amounts of metals as it was used before (Fedorak and Hrudey 1989; Gijzen et al., 2000; Fallon et al., 1991; Paixao et al., 2000; Annachhartre and Amornkaew et al., 2000; Chakarobrty et al., 2005).

First experiment with the second UASB biomass and three different initial concentrations of cyanide (1, 2, 3 mg/L of KCN) completed without noticeable inhibition in comparison to results from a positive control. As the selected biomass originated from UASB reactor treating brewery wastewaters the selected biomass was well adapted to the use of this substrate as carbon source and allowed for quick observation of any kind of inhibition. Free cyanide was quickly disappearing and analyses on all levels showed that that none of cyanide was complexed (measurements of total cyanide at the end of experiment) or escaped via gas phase.

First experiment with granulated biomass from UASB reactor showed more than 80 % of free cyanide removal. This was expected since there were only small amounts of metals in selected UASB biomass (Table 12).

In the second experiment with UASB biomass we used two initial concentrations of KCN; 5.0 and 8.5 mg/L. In the case of cyanide sample with initial concentration 5.0 mg/L methanogenesis was completed in a similar manner as that observed in the positive control. Meanwhile in the sample with higher cyanide concentration, we observed strong inhibition of the process, which lasted for approximately 21 days. After that normal methanogenesis was observed. Interestingly both cyanide samples had produced more biogas after 60 days of incubation at the expense of methane. Cumulative methane production was higher in cyanide-amended samples, suggesting that a fraction of biogas was derived from CN^- degradation probably by hydrolytic pathway.

With respect to the experimental data including short-term aseptic experiment we believe that cyanide was actually removed from the system by the hydrolytic reactions, probably partly spontaneous, abiotic and partly biotic with enzymes of microbial community. This resulted in the formation of simple organic or inorganic molecules, which were further metabolized by microbial consortia as was reported before (Fedorak and Hrudey 1989; Gijzen et al., 2000; Fallon et al., 1991; Paixao et al., 2000; Annachhartre and Amornkaew et al., 2000; Chakarobrty et al., 2005). Based on the results of the study we concluded that anaerobic degradation of cyanide is a combination of simultaneous abiotic and biotic processes and not exclusively biological. In addition, based on the results of the study using CSTR and UASB biomass, we concluded, that anaerobic degradation of cyanide in most environmental conditions where complex environmental conditions exist is most probably a combination of competitive abiotic and biotic processes where dynamically changing conditions at local microscale (< 10 μm) most probably determine the extent of each reaction.

T-RFLP profiling of the microbial community revealed almost the same result as that with CSTR biomass. Bacterial microbial community was more responsive to changes

in the environment than methanogenic archaeal microbial communities, with lesser rearrangements to maintain functionality than observed in CSTR biomass.

Cloning and sequencing revealed that prevalent bacterial phylum in UASB biomass was *Synergistetes* and *Firmicutes*. More interesting were results from cloning and sequencing of positive control and cyanide sample with initial concentration of 8.5 mg/L of KCN. The main difference was in phylum *Firmicutes*. In the cyanide sample *Firmicutes* was the most abundant group 48.2 %, meanwhile in positive control sample was only 19.7 % of *Firmicutes*. This suggests that cyanide degradation was based on bacterial microbial community, since we did not observe any significant difference between initial and developed (positive control and cyanide) with T-RFLP profiling of archaeal community. In addition, the archaeal microbial community was also highly monophyletic and was almost entirely represented by one big class – *Methanobacteria* that is mostly associated with hydrogenotrophic methanogenesis.

Based on the results of cloning and sequencing, we believe that members of the phylum *Firmicutes* are the ones responsible for degradation of intermediate products of the hydrolytic reaction, which takes part in the anaerobic digestion of cyanide. It was reported before that phylum *Firmicutes* was most abundant group in anaerobic digestion of wastewaters, which contained huge amount of ammonia (Yabu et al., 2010). There were also reports on the presence of *Firmicutes* in rather unusual or recalcitrant and hardly degradable substrates for anaerobic digestions (Gray et al., 2010, Abu-Laban et al., 2009 Kniemeyer et al., 2007, Wang et al., 2011). It thus seems that *Firmicutes* are quite adaptive microorganisms, and their role in anaerobic digestion of difficulty degradable substrates should be further investigated by molecular approaches. In addition, their physiological aspects should also be explored in order to determine the environmental factors besides ammonia that control their performance in anaerobic reactors. As it is known, only less than 1% of microbial consortia can be cultivated using classical approaches, however, an increase in cultivation success and detailed physiological studies can be obtained by the use of more modern and group targeting approaches. With further investigations of *Firmicutes* and with their subsequent cultivation, technology of immobilized cells or enzymes from metagenomic approaches could be applied. Cell immobilization is technology in which we entrap or attach a cell to different medium, with flocculation,

covalent bonding to carries, encapsulation in polymer gel, adsorption on surfaces…etc (Lopez et al., 1997). The technology of immobilized cells can be applied in biological treatment to enhance the efficiency of biological degradation. The main advances of cell immobilization are higher concentrations of microorganisms in reactor and protection of cells against toxic substances (Dursun and Tepe 2005), which is very welcome in case of cyanide wastewaters. Chen et al. (2008) demonstrated that immobilized cells could be more tolerant to external shocks including to a higher level of KCN concentration and wider ranges of ambient pH conditions than free suspension cell.

The differences we described in the microbial community structure of archaea based on microarray data did not represent significant changes in microbial structure, a finding congruent with our observations made at the level of phylogenetic profiling using T-RFLP (Chapter 4). This shows that in the future analyses of microbial communities, the sole choice of methodology does not fundamentally affect the experimental outcome and allows one to obtain congruent results by the linking of different methodologies: (i) Microarray vs. T-RFLP (this study) and (ii) Microarray and Q-PCR (Goberna et al., 2010). However, the use of microarrays extended the information we obtained from fingerprinting as trends worth of future exploration were detected. The group of *Methanosarcina* was found to be highly associated with the increased cyanide concentrations, which is also congruent with the fact that this group of archaea is highly resistant to increased ammonia or acetate concentrations. The resistance to increased ammonia concentrations may play the crucial role at the microscale (of granulae) where ammonia is probably formed by *Firmicutes* in the close proximity of archaea thereby allowing the archaea to maintain their functionality throughout the process.

In the future, the experiments with a number of repetitive applications of 8.5 mg/mL cyanide could provide conclusive proof whether this group of archaea is indeed involved in the biogas formation in the presence of ammonia formed from cyanide. From the point of view of biomass selection for the start up of reactors for degradation of cyanide, the UASB biomass would be most suitable as it already contained the microbial communities at bacterial and archaeal levels actively

contributing to degradation of amended cyanide up to the 8.5 mg /L cyanide without previous adaptation period.

These are the three major venues of cyanide degradation that were explored in this thesis out of all possible found in the literature:

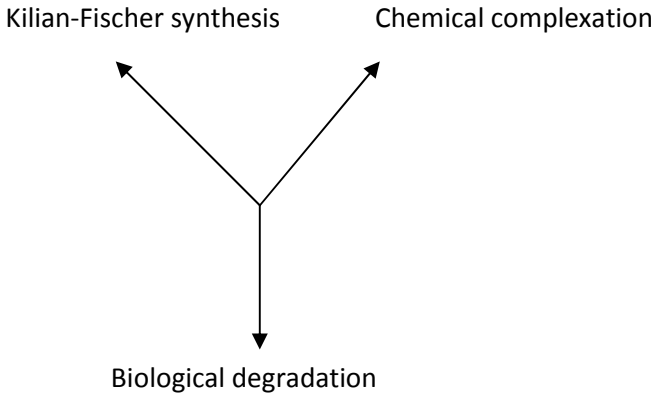

Fig. 61: *three major venues of cyanide degradation*

None of the pathways could be shown to be completely absent from our systems under exploration due to chemical and biological complexity of such determinations; however, the major part of transformations of cyanide observed in our experimental systems using CSTR and UASB biomass could be summarized as following:

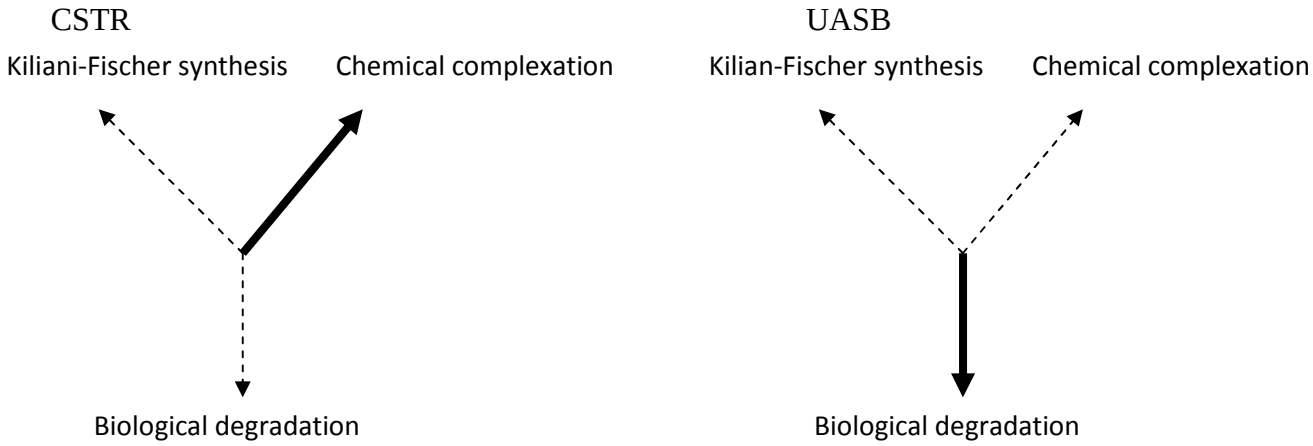

Fig. 62: *suggested major venues of cyanide degradation in CSTR and UASB biomasses based on the findings presented in Chapters 3 and 4.*

We believe that in case of anaerobic digestion competitive abiotic/biotic process takes part and are steered by the trade off between the purely chemical and biological reactions taking place in the same environment at the same time. Cyanide is in our opinion first transformed via hydrolytic reaction to formate (later converted to bicarbonate) and ammonia. Reductive reaction to methane and ammonia is in our opinion less probable since the organisms for such pathway are very rarely found in anaerobic environments. Also there are no reports of occurrence of reductive reaction in anaerobic degradation of cyanide, however, novel molecular tools would need to be designed to follow the extent of this reaction and define the ~~the~~ representatives of that microbial consortia. Thus, further investigations with more powerful techniques such as Stable Isotope Probing (SIP) and Halogen In Situ Hybridization-Secondary Ion Mass Spectroscopy (HISH-SIMS), would in our opinion most probably reveal that key players. SIP is a powerful and comparatively novel technique for identifying microorganisms that are actively involved in specific metabolic processes. It employs substrates that contain heavy isotope of carbon, ^{13}C or nitrogen ^{15}N and is typically used to reveal the diversity behind specific metabolic transformation. In our case labeled KCN ($K^{13}C^{15}N$) would thus serve as cyanide source to biomass in essentially similar incubation experiments as described in this thesis. Then labeled DNA (heavy fraction) can be separated from the unlabelled by standard techniques. Metabolism or incorporation of the ^{13}C or ^{15}N substrates into biomass by organisms would make their DNA isotopically heavier than DNA of those cells that did not incorporate $K^{13}C^{15}N$ derived degradation product making it thus amenable for phylogenetic and functional analyses of genes of interest (Radajewski et al., 2000).

HISH-SIMS (Musat et al., 2008) works on the same principle as SIP. Labeled isotope is added to biomass for incubation period and then filtered through gold-coated filters. Next step is to hybridize 16S rRNA gene with group of specific HRP-labeled oglinonucleotide probes. Results of hybridization is then checked with fluorescence microscopy and applied to nanoSIMS. The NanoSIMS 50L is a nanometer scale secondary ion mass spectrometer with both extremely high lateral resolution and high mass resolution. Because of a unique ion optic design, the primary ion beam can be focused to a very small spot down to less than 50nm beam size and enables simultaneous phylogenetic identification and quantitation of metabolic activities of single microbial cells in the environment (Musat et al., 2008).

In conclusion, in this work we **(i)** established the system for bio-monitoring process of anaerobic degradation by selecting and evaluating suitable experimental methods and used them throughout the study; **(ii)** estimated the effects of a novel and less degradable substrate (brewery spent grain) on bacterial and archaeal microbial communities in three biomasses and **(iii)** determined the possibility of cyanide degradation in two distinct and contrasting biomass samples in relation to previous findings (Gijzen et al., 2000, Fallon et al., 1991, Fedorak and Hrudey 1989). For the precise identification of the key players at the cellular level, different and more complex tools than commonly available to biogas production industry (T-RFLP, cloning and sequencing) should be used (SIP, NanoSims). In this work we described the microbial communities at bacterial and archaeal levels that are involved in the cyanide degradation under anaerobic conditions. We believe that bacterial phylum *Firmicutes* is the crucial microbial group in cyanide degradation whereas archaeal group *Methanosarcina* could be associated with the effects caused by the cyanide degradation for the maintenance of the biogas production.

REFERENCES

Abu Laban, N., Selesi, D., Jobelius, C., Meckenstock, R.U., 2009. Anaerobic benzene degradation by gram-positive sulfate-reducing bacteria. FEMS Microbiology Ecology 68: 300-311.

Ahring, B.K., Sandberg, M., Angelidaki, I. 1995. Volatile fatty acids as indicators of process imbalance in anaerobic digestors. Applied and Environmental Microbiology, 41: 559–565.

Alvarez-Mata, J. 1999. Biological Household Waste Treatment in Europe, second Aalborg International Conference. Resources, Conservation and recycling 17 (1): 67-73

Alonso Álvarez E., Callejón Mochón, E., Jiménez Sánchez, J.C., Ternero Rodríguez, M. 2002. Heavy metal extractable forms in sludge from wastewater treatment plants, Cemosphere, 47 (7): 765:775

Altschul, S.F. Gish, E., Miller, W., Myers E.W., and Lipman, D.J. 1991. Basic local alignment search tool, J. Mol. Biol. 215: 403–410.

Annachhatre, A.P. and Amornkaew, A. 2000. Toxicity and degradation of cyanide in batch methanogenesis, Environmental Technolology, 21: 135-145

Annachhatre, A.P. and Amornkaew,A., 2001. Upflow anaerobic sludge blanket treatment of starch wastewater containing cyanide, Water Environmental Research 73: 622-633.

APHA, 2005. Standard methods for the examination of water and wastewater. Washington, D.C., American Public Helath Association, American Water Works Association, Water Environmental Federation, 21st ed., Washington,

ATSDR, Toxicological profile for cyanide (update), U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, GA, 1997.

ASTM, Designation D 2036-98: Standard test methods for cyanides in water: method A. Total cyanides after distillation, in Annual Book of ASTM Standards, Vol. 11.02, ASTM International, West Conshohocken, PA, 1998.

Ausubel, FM., Brent, R., Kingston, R.E., Moore, D., Seidman, J.G., Smith J.A., and Struhl K. 1999 Current Protocols in Molecular Biology 1–2, Wiley, Chapter 2.1.1, 10.1.4.

Blakemore et al., 1979. R.P. Blakemore, D. Maratea and R.S. Wolf , Isolation and pure culture of a freshwater magnetic spirillum in chemically defined medium. J. Bacteriol. 140 (1979), pp. 720–729.

Borowitz, J.L., Isom, G.E., and Baskin, S.I. 2001. Acute and chronic cyanide toxicity, in Chemical Warfare Agents: Toxicity at Low Levels, Somani, S. and Romano, J., Eds., CRC Press, Boca Raton, FL, p. 301.

Bouallagui, Y. Touhami, R.B. Cheikh and M. Hamdi, 2005. Bioreactor performance in anaerobic digestion of fruit and vegetable wastes, Process Biochemistry 40: 989–995.

Bouzas, A., Ribes, J., Ferrer, J., Seco A. 2007. Fermentation and elutriation of primary sludge: Effect of SRT on process performance. Water Research 41: 747-756.

Braak, C. J. F. ter and Šmilauer, P. 2002. CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (version 4.5).

Chakraborty S., and Veeramani H. 2006. Effect of HRT and recycle ration on removal of cyanide,phenol, thiocyanate and ammonia in an anaerobic-anoxic-aerobic continuous system, Process Biochem. 41: 96–105.

Chen Y., Cheng J.J.,Creamer C.S. 2008. Inhibition of anaerobic digestion process: a review. Bioresource Technology, 99 (10): 4044-64.

Chen, C.Y., Kao, C.M., Chen, S.C. 2008. Application of Klebsiella oxytoca immobilized cells on the treatment of cyanide wastewater, Chemosphere, 71: 133-139

Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37 (Database issue): D141-D145; doi: 10.1093/nar/gkn879.

Dash, R.R. 2009. Cyanide in industrial wastewaters and its removal: A review on biotreatment. Journal of Hazardous Materials, 163: 1–11.

DC, New York, USA. EN ISO 11734. Water Quality – Evaluation of the »Ultimate« Anaerobic Biodegradability of Organic Compounds in Digested Sludge – Method by Measurement of the Biogas Production. 1998, 13.

Demirel B., and Scherer P. 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review, Rev. Environ. Sci. Biotechnol. 7: 173–190.

Dearman, B., Marschner P., and Bentham, R.H. 2006. Methane production and microbial community structure in single-stage batch and sequential batch systems anaerobically co-digesting food waste and biosolids, Applied Microbiology and Biotechnology 69 (5): 589–596.

Deublein, D., Steinhsuser, A. 2008. Biogas from waste and renewable resources, Wiley-VCH: 443 pp.

Delbès C., Moletta R., Godon J.J. 2000 Monitoring of activity dynamics of an anaerobic digester bacterial community using 16S rRNA polymerase chain reaction–single-strand conformation polymorphism analysis. Environmental Microbiology 2: 506–515.

de Bok, F.A.M. 2005. The first true obligately syntrophic propionate oxidizing bacterium, Pelotomaculum schinkii sp. nov., co-cultured with Methanospirillum hungatei, and emended description of the genus Pelotomaculum. International Journal of Systematic and Evolutionary Microbiology. 55: 1697-1703.

DIN 32645. Chemical Analysis - Decision Limit, Detection Limit And Determination Limit Under Repeatability Conditions - Terms, Methods, Evaluation. 2008. 28 pp.

DIN 38402-51 German standard methods for the examination of water, waste water and sludge; general information (group A); calibration of analytical methods, evaluation of analytical results and linear calibration functions used to determine the performance characteristics of analytical methods (A 51). 1986. 23 pp.

Drake, J.A., Huxel G.R., and Hewitt C.L. 1996. Microcosms as models for generating and testing community theory, Ecology 77: 670–677.

Dursun, A.Y., and Tepe, O. 2005. Internal mass transfer effect on biodegradation of phenol by Ca-alginate immobilized Ralstonia eutropha, Journal of Hazardous Materials, B126: 105–111.

Ebbs, S. 2004. Biological degradation of cyanide. Current opinion in Biotechnology, 15: 231-236

Eikmanns, B., Fuchs, G. and Thauer, R.K., 1985. Formation of carbon monoxide from CO2 and H2 by Methanobacterium thermoautotrophicum. Eur. J. Biochem. 146, pp. 149–159.

Epstein J. 1947. Estimation of microquantities of cyanide. Anal Chemistry, 19: 272-274.

European Commission Environment, 2011. Biodegradable waste, http://ec.europa.eu/environment/waste/compost/index.htm, accessed; 17.3.2011

Fallon R. D., Cooper D. A., Speece R. and Henson M. 1991. Anaerobic biodegradation of cyanide under methanogenic conditions. Applied Environmental Microbiology 57: 1656 - 1662.

Fallon, R.D. 1992. Evidence of a hydrolytic route for anaerobic cyanide degradation. Apernational and Environmental Microbiology, 58: 3163–3164.

Fan, LI., Jinren NI., Wua, Y., Zhang, Y. 2008. Treatment of bromoamine acid wastewater using combined process of micro-electrolysis and biological aerobic filter. Journal of Hazardous Materials, 162: 1204–1210.

Fedorak, P. M., and S. E. Hrudey. 1989. Cyanide transformation in anaerobic phenol degrading methanogenic cultures. Water Science Technology 21:67-76.

Fernandez-Cegri, M. A. de la Rubia, B. Rincon, M. C. Portillo, J. M. Gonzalez, R. Borja, Influence of the inoculum source and characteristics on the methane yield coefficient, kinetics and microbial communities involved in the anaerobic digestion process of sunflower oil cake in batch mode. 2nd International Conference of IAMAW 2010: http://www. iamawaste.org/wordpress/izmir/pdf/Borja_IAMAW_Izmir.p df (assessed: December 16, 2010)

Fernandez A., Huang S., Seston S., Xing J., Hickey R., Criddle C., Tiedje J. 1999. How stable is stable? Function versus community composition. Applied and Environmental Microbiology, 65: 3697-3704.

Fernandez AS., Hashsham SA., Dollhopf SL., Raskin L., Glagoleva O., Dazzo FB., Hickey RF., Criddle CS., Tiedje JM. 2000. Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Applied and Environmental Microbiology, 66: 4058-4067.

Fechter, L.D., Chen, G.P., and Johnson, D.L. 2002. Potentiation of noise-induced hearing loss by low concentrations of hydrogen cyanide in rats, Toxicol. Sci., (66): 131-138

Foster-Carneiro, T., Perez, M.,Romero, L.I. 2008. Thermophilic anaerobic digestion of source-sorted fraction of municipal solid waste. Bioresource Technology, 99 (14): 6763-6770

Forster-Carneiro, T., Pérez García, M., Romero García, L.I. 2007. Compostingpotential of different inoculum sources on modified SEBAC system treatment of municipal solid wastes. Bioresource Technology, 98 (17): 3354–3366.

Forster-Carneiro, T., Pérez García, M., Romero García, L.I., Sales, D. 2007. Dry thermophilic digestion of organic fraction of the municipal solid waste: focusing on the inoculum sources. Bioresource Technology, 98 (17): 3195–3203.

Franke-Whittle, I.H., Goberna, M., Pfister, V., Insam H. 2009. Design and development of the ANAEROCHIP microarray for investigation of methanogenic communities, Journal of Microbiological Methods 79: 279–288

Garcia J.L., Patel B.K.C., Ollivier B. 2000. Taxonomic phylogenetic and ecological diversity of methanogenic Archaea. Anaerobe, 6 (4): 205-226

Garrity G.M., and Holt J.G. 2001) The Chloroflexi. Bergey's Manual of Systematic Bacteriology (BooneDR & CastenholzRW, eds), Springer-Verlag, New York, pp. 426–446.

Gee, D. 1987. Cyanides in murder,suicide and accident, in Clinical and Experimental Toxicology of Cyanide, Ballantyne, B. and Marrs, T., Eds., IOP Publishers, Bristol, England, p. 209.

Ghosh, R.S., Dzombak, D.A., Luthy, R.G., and Nakles, D.V., Subsurface fate and transport of cyanide species at a manufactured-gas plant site, Water Environmetal Research 71: 1205, 1999.

Gijzen HJ., Bernal E., Ferrer H. 2000. Cyanide toxicity and cyanide degradation in anaerobic wastewater treatment. Water Research, 34: 2447–2454.

Goberna M, Gadermaier M, García C, Wett B, Insam H. 2010. Adaptation of methanogenic communities to the cofermentation of cattle excreta and olive mill wastes at 37°C and 55°C. Applied Environmental Microbiology 76: 6564–6571

Godon, J. J., Moriniere, J., Moletta, M., Gaillac, M., Bru, B., and Delgenes, J.P. 2005. Rarity associated with specific ecological niches in the bacterial world: the "Synergistes" example. Environ. Microbiol. 7: 213-224.

Gray, N.D., Sherry, A., Hubert, C., Dolfing, J., Head, I.M. 2010 Methanogenic degradation of petroleum hydrocarbons in subsurface environments: remediation, heavy oil formation, and energy recovery. In: Allen I. Laskin, Sima Sariaslani and M.G. Geoffrey, Editors, Advances in Applied Microbiology, Academic Press, Burlington, pp. 137–161

Grosskopf, P.H. Janssen and W. Liesack. 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval, Applied and Environmental Microbiology 64: 960–969.

Grotenhuis, M. Smit, C.M. Plugge, Y.S. Xu, A.A. van Lammeren, A.J. Stams and A.J. Zehnder. 1991. Bacteriological composition and structure of granular sludge adapted to different substrates, Applied Environmental Microbiology 57: 1942–1949

Grüntzig, V., Stres, B., Ayala del Río, H.L., Tiedje, J.M. 2002. Improved protocol for T-RFLP by capillary electrophoresis. East Lansing: Michigan State University, Center for Microbial Ecology, 1 - 4.

Gupta N., Balomajumder C., Agarwal V.K. 2010. Enzymatic mechanism and bio-chemistry for cyanide degradation: A review. Journal of Hazardous Materials 176 (1-3): 1-13.

Hashsham, S.A., A.S. Fernandez, S.L. Dollhopf, F.B. Dazzo, R.F. Hickey, J.M. Tiedje and C.S. Criddle. 2000. Parallel processing of substrate correlates with

greater functional stability in methanogenic bioreactor communities perturbed by glucose. Applied Environmental Microbiology, 66:4050-4057.

Hedderich R., Whitman W.B. 2006. Physiology and biochemistry of the methane-producing Archaea. In: The Prokaryotes: Vol. 2: Ecophysiology and biochemistry. Dworkin M., Falkow S., Rosenberg E., Schleifer K.-H., Stackebrandt E. (eds.). New York, Springer: 1050-1079

Holdeman, L. V., Cato, E. P., Moore, W. E. C. 1977. Anaerobe Laboratory Manual. 4th Edition, VPI, Blacksburg, Virginia 1–156.

Hope, K.M., Knowels, C.J. 1991. The anaerobic utilization of cyanide in the presence of sugars by microbial cultures can involve abiotic process, FEMS Microbiology Letters 80: 217:220

Hori T., Haruta S., Ueno Y., Ishii M., Igarashi Y. 2006. Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. Applied and Environmental Microbiology, 72: 1623-1630.

Hua L., Wu, W.X., Liu, Y.X., Tientchen C.M., Chen Y.X. 2008. Heavy Metals and PAHs in Sewage Sludge from Twelve Wastewater Treatment Plants in Zhejia, Biomedical and Environmental Sciences, 21 (4): 345-352.

Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., Lens, P.N.L. 2004. Anaerobic sludge granulation, Water Research, 38: 1376–1389

Imachi, H., Y. Sekiguchi, Y., Kamagata, A., Ohashi, P., Harada, H. 2000. Cultivation and in situ detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. Applied and Environmental Microbiology, 66: 3608-3615.

Ishii, S., Kosaka, T., Hori, K., Hotta, Y., Watanabe, K. 2005. Coaggregation Facilitates Interspecies Hydrogen Transfer between Pelotomaculum thermopropionicum and Methanothermobacter thermautotrophicus. Applied and Environmental Microbiology, 71: 7838-7845.

James, J., and Boegli, L. 2001. Degradation of cyanide and metal–cyanide complexes by bacterial consortia, in Cyanide: Social, Industrial and Economic Aspects, Young, C.A., Twidwell, L.G., and Anderson, C.G., Eds., The Minerals, Metals and Materials Society, Warrendale, PA, 223 pp.

Jessup, C. M., R. Kassen, S. E. Forde, B. Kerr, A. Buckling, P. B. Rainey, and B. J.M. Bohannan. (2004) Big questions, small worlds: microbial model systems in ecology. Trends in Ecology and Evolution, 19: 189 – 197.

Jetten, M.S.M., Stams A.J.M., and Zehnder A.J.B. 1992. Methanogenesis from acetate: comparison of the acetate metabolism in Methanothrix soehngenii and Methanosarcina spp. FEMS Microbiol. Rev. 88: 181–198.

Jumas-Bilak, E., Roudière, L., and Marchandin, M. 2009. Description of 'Synergistetes' phyl. nov. and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes', Int. J. Systc. Evol. Microbiol. 59: 1028–1035.

Kittis, C.L. 2001. Terminal restriction patterns: a tool for comparing microbial communities and assessing community dynamics. Current Issues in Intestinal Microbiology, 2: 17-25.

Kniemeyer, O., Musat, F., Sievert, S.M., Knittel, K., Wilkes, H., Blumenberg, M., Michaelis, W., Classen, A., Bolm, C., Joye, S.B., Widdel, F., 2007. Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria. Nature 449: 898-901.

Kobayashi, T., Li Y.Y., Harada H. 2008. Analysis of microbial community structure and diversity in the thermophilic anaerobic digestion of waste activated sludge. Water Science & Technology, 57 (8): 1199-1205.

Krakat, N., Schmidt, S., Scherer P. 2011. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage Bioresource Technology, 102 (10): 5692-5701.

Krakat, N., Schmidt, S., Scherer P. 2010 The mesophilic fermentation of renewable biomass – does hydraulic retention time regulate diversity of methanogens?, Applied Environmental Microbiology, 76 (18): 6322–6326.

Kunapuli, U., Lueders, T., Meckenstock, R.U., 2007. The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. The ISME Journal 1: 643-653.

Kunz, D.A., Nagappan, O., Silva-Avalos, J., and Delong, G.T. 1992. Utilization of cyanide as a nitrogenous substrate by Pseudomonas fluorescens NCIMB 11764: evidence for multiple pathways of metabolic conversion, Applied Biochemiatry and Microbiology, 58: 2022-2029.

Laha, S. and Luthy, R.G.1991. Investigation of microbial degradation of fixed cyanide, report to the Aluminum Association by Carnegie Mellon University, Pittsburgh, PA.

Lee, M., Hidaka, T., Tsuno, H. 2008. Effect of temperature on performance and microbial diversity in hyperthermophilic digester system fed with kitchen garbage. Biosource Technology, 99: 6852-6860.

Lee, C. Kim, J., Shin, S.G., O'Flaherty, V., Hwang S. 2010. Quantitative and qualitative transitions of methanogen community structure during the batch anaerobic digestion of cheese-processing wastewater, Applied Microbiology and Biotechnology, 87: 1963–1973.

Leiu F., Hua W., Yinguang C., Qin W. 2009. Effect of solids retention time and temperature on waste activated sludge hydrolysis and short-chain fatty acids accumulation under alkaline conditions in continuous-flow reactors. Biosource Technology, 1: 44-49.

Likar, K., M. Regvar, I. Mandi}-Mulec, B. Stres, H. Bothe. 2009. Diversity and seasonal variations of mycorrhiza and rhizosphere bacteria in three common plant species at the Slovenian Ljubljana Marsh Biological and Fertility of Soils, 45: 573–583.

Liu, W-T., Marsh, T.L., Cheng, H., Forney, L.J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology, 63: 4516-4522.

Lopez, A., Lazaro A., and Marques, N. 1997 The interphase technique—a simple method of cell immobilization in gel-beads, J. Microbiol. Meth. 30 (1997), pp. 231–234.

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.-H., Wagner, M., 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Applied Environmental Microbiology 68: 5064–5081.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Yadhukumar, H.M., Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, G., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer. K.H. 2004. ARB: a software environment for sequence data. Nucleic Acids Research, 32 (4): 1363-1371.

Madigan, T.M., Martinko, J.M., Dunlap, P.V., Clark, D.P., Brock T. 2009. Brock Biology of Microorganisms, 12th edition. Pearson education Inc., 1061 pp.

McHugh, M. C., Collins G., and O'Flaherty V. 2006. Reactor performance and microbial community dynamics during anaerobic biological treatment of wastewater at 16–37 °C, FEMS Microbiol. Ecol. 48: 369–378

McLeod F.A., Guiot S.R., Costerton J.W. 1990. Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor, Applied Environmental Microbiology 56(6): 1598–607.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J., Schink, B., Rohlin, L., Gunsalus, R.P. 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Annals of the New York Academy of Sciences, 1125: 58–72.

McMahon, K.D., Stroot, P.G., Mackie, R.I. Raskin, L. 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—II: microbial population dynamics. Water Research, 35: 1817–1827.

McRae, D.G., Coker, J.A., Legge, R.L. and Thompson J.E. 1983. Bicarbonate/CO2-Facilitated Conversion of 1-Amino-cyclopropane-1-carboxylic Acid to Ethylene in Model Systems and Intact Tissues, Plant Physiology, 73 (39): 784-790.

Meeussen, J.L., Keizer, M.G., and de Haan, F.A.M. 1992. Chemical stability and decomposition rate of iron cyanide complexes in soil solutions, Environmental Science and Technology, 26: 511-516.

Montero, B., Garcia-Morales, J.L., Sales, D., Solera, R. 2007. Evolution of microorganisms in thermophilic-dry anaerobic digestion. Bioresource Technology, 99: 3233-3243.

Musat, N., Halm, H., Winterholler, B., Hoppe, P., Peduzzi, S., Hillion, F., Horreard, F., Amann, R., Jorgensen, B. B. and Kuypers, 2008. A single-cell view on the ecophysiology of anaerobic phototrophic bacteria. Proc Natl Acad Sci USA 105: 17861–17866

Mussatto S.I., Dragone G., and Roberto I.C. 2006. Brewer's spent grain: generation, characteristics and potential applications, J. Cereal Sci. 43: 1–14.

Narihiro T., Sekiguchi Y. 2007. Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. Current Opinion in Biotechnology, 18 (3): 273-278.

Nyberg, L.; Turco, R.F.; Nies, L. 2008. Assessing the Impact of Nanomaterials on Anaerobic Microbial Communities. Environmental Science and Technology, 42: 1938-1943.

Novak, D., Stres, B., Osojnik, G., Škrjanec I., and Marinšek Logar, R. 2011. General Microbial Community Flexibility in Biochemical Methane Potential Assay is Highly Correlated to Initial Biogas Production Rates. Acta Chim. Slov., 58: 171–175.

Osborne, A.M., Moore, E.R.B., Timmis, K.N., 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology. 2: 39–50.

Owen W.F. , D.C. Stuckey, J.B. Healy, L.Y. Young and P.L. Mc Carty, Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Research 13 (1979), pp. 485–492.

Paixao, M.A., Tavares, C.R.G., Bergamasco, R., Bonifacio, A.L.E., and Costa, R.T. 2000. Anaerobic digestion from residue of industrial cassava industrialization

with acidogenic and methanogenic physical separation phases, Appl. Biochem. Biotech., 84–86: 809-819.

Palatsia J., Viñasa M., Guivernaua M., Fernandeza B., and Flotats X. 2011. Anaerobic digestion of slaughterhouse waste: Main process limitations and microbial community interactions, Bioresource Technology, 102 (3): 2219-2227.

Pavlakovic, G., Rathinavelu, A., and Isom, G.E. 1994. MK-801 prevents cyanide-induced changes of Fos levels in rat brain, Neurochem. Res., (19): 1289, 1994.

Plugge, C.M. 2002. Desulfotomaculum thermobenzoicum subsp. thermosyntrophicum subsp. nov., a thermophilic, syntrophic, propionate-oxidizing, spore-forming bacterium. International Journal of Systematic and Evolutionary Microbiology. 52, 391-399.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic acids research, 35(21): 7188-96.

Quan, ZX., Bae JW., Rhee. SK., Cho, YG., Lee, ST. 2004. Toxicity and degradation of metal-complexed cyanide by a bacterial consortium under sulfate-reducing conditions. Biotechnology Letters, 26: 1007-1011.

Radajewski, S., Ineson, P., Parek, N.R., and Murrell J.C. 2000. Stable-isotope probing as a tool in microbial ecology, Nature 403: 646-649

Raybuck, S.A. 1992. Microbes and microbial enzymes for cyanide degradation. Biodegradation, 3: 3-18.

Richardson RE., Bhupathiraju VK., Song DL., Goulet TA., Alvarez-Cohen L. 2002. Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. Environmental Science and Technology, 36: 2652–2662.

Rincón, B., Raposo, F., Borja, R., González, J.M. Portillo, M.C., and Saiz-Jimenez, C. 2006. Performance and microbial communities of a continuous stirred tank reactor treating two-phases olive mill solid wastes at low organic loading rates, J. Biotechnol. 121 (4): 534–543.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S Guermazi, S., Weissenbach, J., Camacho T.L.P., and Sghir, A. 2009 Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge, ISME J. 3 (6): 700–714.

Rollinson G., Jones, R., Meadows, M.P., Harris, R.E., Knowles C.J. 1987. The growth of a cyanide-utilising strain of Pseudomonas fluorescens in liquid culture on nickel cyanide as a source of nitrogen, FEMS Microbiology Letters, 40 (2-3): 199-205.

Schecher, W.D. and McAvoy, D.C., MINEQL+: a chemical equilibrium modeling system, Ver. 4.0 for Windows, Environmental Research Software, Hallowell, ME, 1998.

Schnurer A., Zellner G., Svensson B. H. 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiology. Ecology, 29: 249-261.

Sehmel, G.A. 1989. Cyanide and antimony thermodynamic database for the aqueous species and solids for the EPA-MINTEQ geochemical code, PNL-6835, Pacific Northwest Laboratory, Richland, WA.

Sekiguchi Y. 2006. Yet-to-be cultured microorganisms relevant to methane fermentation processes, Microbes Environ. 21 (1): 1–15.

Sharpe, A.G. 1976 Chemistry of Cyano Complexes of the Transition Metals, Academic Press, London, 313 pp.

Shigematsu T, Tang YQ, Mizuno Y, Kawaguchi H, Morimura S, Kida K. 2006. Microbial diversity of a mesophilic methanogenic consortium that can degrade long-chain fatty acids in chemostat cultivation. Journal of Bioscience and Bioengineering 102: 535–544.

Shin S.G., Han, G., Lim, J. Lee, C. and Hwang S. 2010. A comprehensive microbial insight into two-stage anaerobic digestion of food waste-recycling wastewater, Water Research, 44 (17): 4838-4849.

SIST EN ISO 11734. Water Quality – Evaluation of the »Ultimate« Anaerobic Biodegradability of Organic Compounds in Digested Sludge – Method by Measurement of the Biogas Production. 1998, 13 pp.

SIST EN ISO 8466. Water quality -- Calibration and evaluation of analytical methods and estimation of performance characteristics -- Part 1: Statistical evaluation of the linear calibration function. 1990, 24 pp.

Smith, M.R., Lequerica, J.L. and Hart, M.R., 1985. Inhibition of methanogenesis and carbon metabolism in Methanosarcina sp. by cyanide. J. Bact. 16: 267–71.

Stres, B., T. Danevcic, L. Pal, M. M. Fuka, L. Resman, S. Leskovec, J. Hacin, D. Stopar, I. Mahne, and I. Mandic-Mulec. 2008. Influence of temperature and soil water content on bacterial, archaeal and denitrifying microbial communities in drained fen grassland soil microcosms. FEMS Microbiology Ecology, 66:110-122.

Stres, B., Tiedje, J. M., Murovec, B. (2009). BEsTRF: a tool for optimal resolution of terminal-restriction fragment length polymorphism analysis based on user-defined primer-enzyme-sequence databases. Bioinformatics 25: 1556-1558

Stres, B., L. Philippot, J. Faganeli, J. M. Tiedje J.M. 2010. Frequent freeze–thaw cycles yield diminished yet resistant and responsive microbial communities in

two temperate soils: a laboratory experiment, FEMS Microbiology ecology, 74: 323–335.

Vartoukian, S.R., Palmer, R.M., and W. G. Wade. 2009. Diversity and Morphology of Members of the Phylum "Synergistetes" in Periodontal Health and Disease. Applied and Environmental Microbiology, 75 (11): 3777-3786.

Vartoukian SR, Palmer RM, Wade WG. 2007. The division 'Synergistes'. Anaerobe 13: 99–106.

Voorthuizen, E., Zwijnenburg, A., Meer, V., Temmink, H. 2008. Biological black water treatment combined with membrane separation. Water Research 42: 4334-4340.

Wang L.Y., Gao C.X.,, Mbadinga S.M.,, Zhou L.,, Liu, J.F., Gu, J.D., and Zhong B. 2010. Characterization of an alkane-degrading methanogenic enrichment culture from production water of an oil reservoir after 274 days of incubation, International Biodeterioration & Biodegradation, 65 (3): 445-450.

Wang, Q, G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Applied Environmental Microbiology, 73 (16): 5261-7.

Ward A.J., Hobbs. P.J., Holliman, P.J. and Jones, D.L. 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresearch Technology 99: 7928-7940.

Watanabe, T., Asakawa S., Nakamura, A., Nagaoka, K., Kimura, M. 2004. DGGE method for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil. FEMS Microbiology letters 232 (2): 153-163

Wild, S.R., Rudd, T., Neller, A. 1994. Fate and effects of cyanide during wastewater treatment processes. Science of the Total Environment, 156: 93–107.

Wu, H., Jiang, Z.Y, Xu, S.W, 2003. Conversion CO2 to formate by formate dehydrogenase immobilized in silicate matrix. Prepr.Pap-Am. Chem.Soc., Div.Fuel Chem 48: 630

Yabu, H., Sakai, C., Fujiwara, T., Nishio, N., and Nakashimada , Y. 2010. Thermophilic two-stage dry anaerobic digestion of model garbage with ammonia stripping, Journal of Bioscience and Bioengineering, 111 (3): 312-319.

Yamada T., Sekiguchi, Y., Imachi H., Kamagata Y., Ohashi A., and Harada H. 2005. Diversity, localization, and physiological properties of filamentous microbes belonging to Chloroflexi subphylum I in mesophilic and thermophilic methanogenic sludge granules, Applied and Environmental Microbiology 71 (11): 7493–7503.

Yang, J., Speece, R.E., Parkin, G.F., Gosset, J. and Kocher, W., 1980. The response of methane fermentation to cyanide and chloroform. Prog. Water Tech. 12: 977–988.

Zarzycki J., Brecht V., Müller M., and Fuchs G. 2009. Identifying the missing steps of the autotrophic 3-hydroxypropionate CO2 fixation cycle in Chloroflexus aurantiacus, Proc. Natl. Acad. Sci. USA 106 (50): 21317–21322.

Zinder S.H., 1993. Physiological ecology of methanogens. In: J.G. Ferry, Editor, Methanogenesis: Ecology, Physiology, Biochemistry and Genetics, Chapman & Hall, New York, pp. 128–206.

ANNEX 1

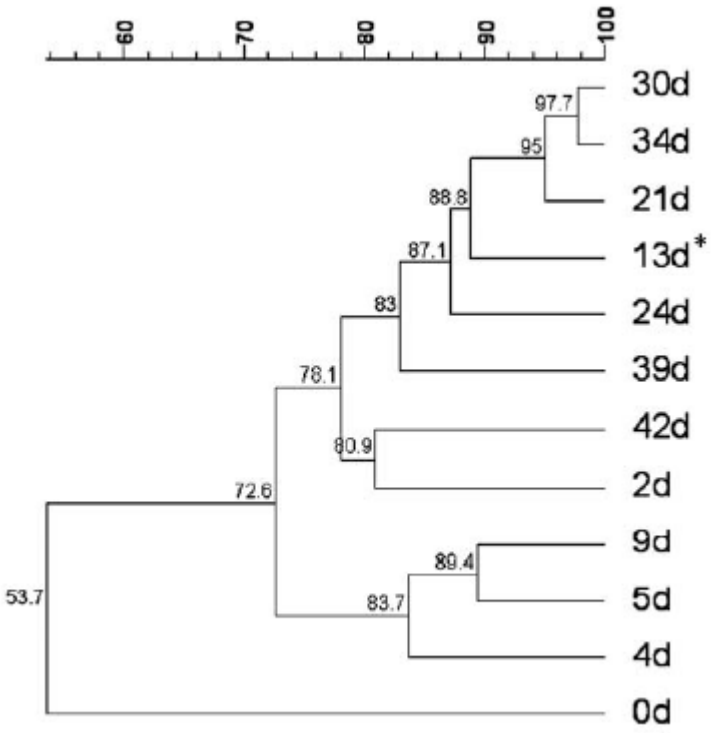

Figure A1: A reanalysis of archaeal microbial community dynamics during 42 day batch start-up of digester on a single substrate (Lee et al., 2010). * – time of incubation comparable to 14 days used in routine biochemical methane potential assays. Three separate lineages could be delineated by cluster cut-off method (BioNumerics, Applied- Maths). For the exploration of microbial community dynamics the use of other substrates (or absence of any) was not reported.

ANNEX 2

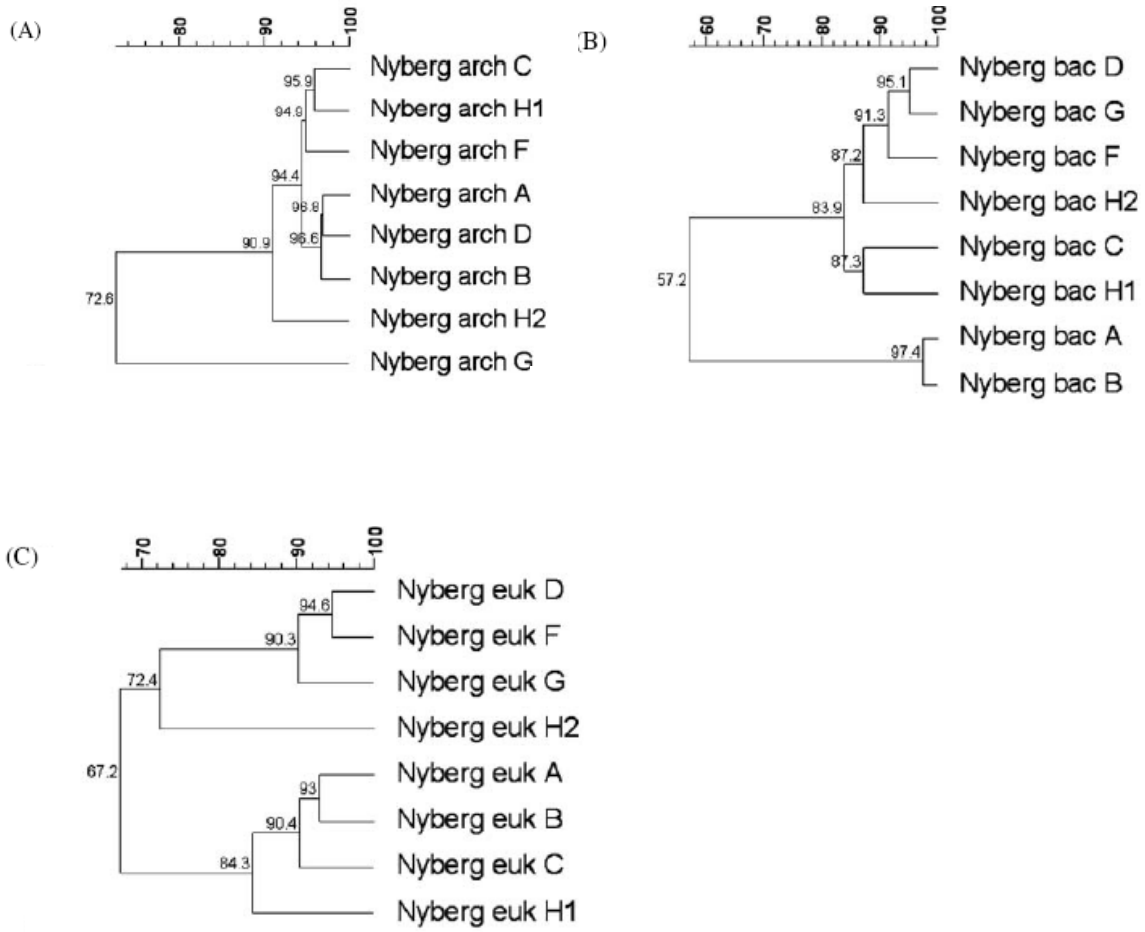

Figure A2: A reanalysis of the developed archaeal (A), bacterial (B) and eukaryotic (C) microbial communities at the end of degradation test different organic compounds in BMP (Nyberg et al., 2008). The data on the initial communities present in live or dried sludge were not provided.

In archaeal communities (A), only in treatment G (live sludge + dried sludge) significant differences could be detected, whereas all other developed communities were highly similar. It is important to note that only archaeal 16S rRNA genes were amplified using nested PCR design.

In bacterial communities (B), developed communities in treatments A (live sludge + fullerene dissolved in methanol) and B (live sludge + water solution of fullerene) developed significantly different communities from reference samples (H1 and H2).

In eukaryotic microbial communities (C), three separate clusters could be detected. First, all communities developed in the presence of fullerene formed a distinct cluster separated from those communities developed in the presence of dried sludge with or without toluene or xylene. In addition, both clusters were significantly different from reference H2, containing only live sludge.

ANNEX 3

Table A3. List of oligonucleotide probes and target organisms included in the microarray (Frank-Whittle et al., 2009).

Probe name	**Target organism**	**Probe sequence 5′–3′**	***E. coli* position**	**Percent GC**	**Melting temp [℃]**
Arc917	Archaea	TTTTTTTTTTTTGUGCUCCCCCGCCAAUUC	917	60	67.5
Mbac855	Methanobacteriaceae	TTTTTTTTTTTTCTTAACAGCTTCCCTTCGG	855	52.6	59.7
Mbac313	Methanobacteriaceae	TTTTTTTTTTTTACCTTGTCTCAGGTTCCATC	313	50	57.6
MMMM817	*Methanothermobacter*, *Methanobacterium*, *Methanosphaera*, *Methanobrevibacter*	TTTTTTTTTTTTCAACACCAAGTCCACATCG	817	52.6	58.3
MbM405	*Methanobacterium*, *Methanothermobacter*	TTTTTTTTTTTTACCCCGTTAAGAGTGGC	405	58.8	56.4
MlM594	*Methanolobus*, *Methanomethylovorans*	TTTTTTTTTTTTCAGATTTCCCGGAAGACTG	594	52.6	59.4
MMM309	*Methanomicrobium*, *Methanogenium*, *Methanoplanus*	TTTTTTTTTTTTTGTCTCAGATTCCATCTCCG	309	50	59.4
MMbac183	*Methanobacterium*	TTTTTTTTTTTTTAGGGAACCAATTCCAGG	183	50	56.9
MMbac624	*Methanobacterium*	TTTTTTTTTTTTAUCUCCAGCAAUUCCACAC	624	47.4	56.4
Mbc263	*Methanobacterium*	TTTTTTTTTTTTACTGGCTTGGTGGGCAT	263	58.8	59.9
Mbc542	*Methanobacterium*	TTTTTTTTTTTTAAAATGGCCACCACTTGAG	542	47.4	59.9
Mbc644	*Methanobacterium*	TTTTTTTTTTTTCCGGCCTCAAGCCTAAT	644	58.8	60.0
Mbc744	*Methanobacterium*	TTTTTTTTTTTTTACTCACCGTCAGGTCCG	744	61.1	57.7
MbcF733	*Methanobacterium formicicum*	TTTTTTTTTTTTGGCCCGTTCCAGTTAGC	733	64.7	59.9

MbcF745	*Methanobacterium formicicum*	TTTTTTTTTTTTTTACTCACCGTCAGGCCC	745	61.1	60.0
Mbre364	*Methanobrevibacter*	TTTTTTTTTTTTTTCTCACATTGCGGAGGT	364	50	57.6
Mbre377	*Methanobrevibacter*	TTTTTTTTTTTTCCCGTCGCGATTTCTC	377	62.5	58.2
Mbre406	*Methanobrevibacter*	TTTTTTTTTTTTCCATCCCGTTAAGAATGG	406	50	57.5
Mbre846	*Methanobrevibacter*	TTTTTTTTTTTTCCCTTCGGCACTGAAAC	846	58.8	57.6
MbreS840	*Methanobrevibacter smithii*	TTTTTTTTTTTTACTGGGACAGCTCAAAGC	840	55.6	56.5
MbreS731	*Methanobrevibacter smithii*	TTTTTTTTTTTTAGAATCGTTCCAGTCAGACG	731	50	59.0
MbreS178	*Methanobrevibacter smithii*	TTTTTTTTTTTTCATTACAGGAATAATTGCCTAT	178	31.8	56.9
Mcal465	*Methanocalculus*	TTTTTTTTTTTTCCCTTTCTCCTGGTGTGTGT	465	55	61,1
Mcal585	*Methanocalculus*	TTTTTTTTTTTTCCCAGAGACTTAACGACCAA	585	50	59,5
Mcal600	*Methanocalculus*	TTTTTTTTTTTTGCCAAAAGATTTCCCCAG	600	50	59,6
Mcal840	*Methanocalculus*	TTTTTTTTTTTTCACCTCAGCGACTCGTGG	840	66.7	61.1
Mcal422	*Methanocalculus*	TTTTTTTTTTTTACACCAGAACAGCCTGCA	422	55.6	58.1
Mcal707	*Methanocalculus*	TTTTTTTTTTTTTCGGTCGTCCCTCGG	707	73.3	58.1
Mcald179	*Methanocaldococcus*	TTTTTTTTTTTTTCCAGACCTCCTCCCCTA	179	61.1	58.4
Mcald276	*Methanocaldococcus*	TTTTTTTTTTTTGCCCGTACGGATCGTAG	276	64.7	58.1
Mcald444	*Methanocaldococcus*	TTTTTTTTTTTTCCCGGAGCTGTTTACACT	444	55.6	57.0
Mcald822	*Methanocaldococcus*	TTTTTTTTTTTTCGACGCGACACCTAGTCC	822	66.7	60.6
Mcoc139	*Methanococcoides*	TTTTTTTTTTTTCGGAGTTATGCCGAACCT	139	55.6	60.0
Mcoc194	*Methanococcoides*	TTTTTTTTTTTTCTTAGGCAACCGAAGTCTTT	194	45	59
Mcoc438	*Methanococcoides*	TTTTTTTTTTTTTGCCGTTTACACATGTGGA	438	47,4	59.7
Mcoc465	*Methanococcoides*	TTTTTTTTTTTTCTTGCTAACACATGCCGTT	465	47.4	58.7

Mcoc584	*Methanococcoides*	TTTTTTTTTTTTGAAGAACTGATCAAACCGG	584	47.4	56.6
Msco183	*Methanocorpusculum*	TTTTTTTTTTTTCCATTCCAGGACATATCCA	183	47.4	57.3
Msco583	*Methanocorpusculum*	TTTTTTTTTTTTCAAGAGACCTAACAGTCAAGC	583	47.6	56.3
Msco832	*Methanocorpusculum*	TTTTTTTTTTTTTAACACGTGGTTACCGACA	832	47.4	56.4
Msco843	*Methanocorpusculum*	TTTTTTTTTTTTTCGGCGCATCAGTAACAC	843	55.6	59.4
Mcul574	*Methanoculleus*	TTTTTTTTTTTTAACCCAGCTACGAACGC	574	58.5	57.8
Mcul170	*Methanoculleus*	TTTTTTTTTTTTGCATCTGTAACCTATAGGGTATT	170	39.1	57.9
Mcul302	*Methanoculleus*	TTTTTTTTTTTTTCCAACTCCGGGCTCTT	302	58.8	59.4
Mcul393	*Methanoculleus*	TTTTTTTTTTTTTACAGGCACTCGAGGTTCC	393	57.9	59.6
Mcul669	*Methanoculleus*	TTTTTTTTTTTTCCCCCGAAGTACCCCT	669	68.8	58.5
Mg193	*Methanogenium*	TTTTTTTTTTTTTATGGCGCCTCAACTTTC	193	50	58.4
Mg229	*Methanogenium*	TTTTTTTTTTTTCTAATCGACCGCAGATCC	229	55.6	57.3
Mg619	*Methanogenium*	TTTTTTTTTTTTTAGACGCCCGTCGGTTA	619	58.5	59.2
Mg833	*Methanogenium*	TTTTTTTTTTTTAGTGACTCGTGGTCACCAGT	833	55	58.2
Mhal183	*Methanohalobium*	TTTTTTTTTTTTACCACAGAGCCTTCCAGC	183	61.1	59.0
Mhal236	*Methanohalobium*	TTTTTTTTTTTTCACTACCACCTGATAGACCG	236	55	56.9
Mhal598	*Methanohalobium*	TTTTTTTTTTTTCACCAGATTTCCCAATGG	598	50	57.5
Mhal829	*Methanohalobium*	TTTTTTTTTTTTGCCGTACCATCCCTAACAC	829	57.9	59.4
Mlob133	*Methanolobus*	TTTTTTTTTTTTTATGCCTGACCTTAGGGC	133	55.6	57.4
Mlob183	*Methanolobus*	TTTTTTTTTTTTCACAGAACATTCCAGTATCTATGA	183	37.5	59.2
Mlob389	*Methanolobus*	TTTTTTTTTTTTGGCACTCAGTATCCCCTTA	389	52.6	56.5
Mlob821	*Methanolobus*	TTTTTTTTTTTTGTCCCAGACACCTAGCGAG	821	63.2	59.1
Mmic183	*Methanomicrobium*	TTTTTTTTTTTTAGGAGACATTCCAGTACTCCT	183	47.6	56.5

Mmic615	*Methanomicrobium*	TTTTTTTTTTTTACGCCTCACAGTTAAGCC	615	55.6	57.2
Mmic829	*Methanomicrobium*	TTTTTTTTTTTTAACTCGTAGTTACAGGCACAC	829	47.6	56.7
Mmic854	*Methanomicrobium*	TTTTTTTTTTTTTCACGTTTTCACTTCGGC	854	50	58.6
Msae406	*Methanosaeta*	TTTTTTTTTTTTAGCCAGATTTGTAACCTGG	406	47.4	56.8
Msae827	*Methanosaeta*	TTTTTTTTTTTTACCGTGGCCGACACCT	827	68.8	60.0
Msae841	*Methanosaeta*	TTTTTTTTTTTTCACCGACAACGGTCGC	841	68.8	59.3
Msar184	*Methanosarcina*	TTTTTTTTTTTTGACGCATAAAGCATTCCAG	184	47.4	58.4
Msar197	*Methanosarcina*	TTTTTTTTTTTTCTTGGGCAGACGAATCC	197	58.8	57.3
Msar549	*Methanosarcina*	TTTTTTTTTTTTCCCAATAATCACGATCACC	549	47.4	57.2
Msar587	*Methanosarcina*	TTTTTTTTTTTTCCGGAGGACTGACCAAAC	587	61.1	59.2
Msar416	*Methanosarcina*	TTTTTTTTTTTTCTGGACAGCCAGCATATGA	416	52.6	58.5
Msar601	*Methanosarcina*	TTTTTTTTTTTTAGCTATCAGATTTCCCGG	601	50	56.5
Msph125	*Methanosphaera*	TTTTTTTTTTTTCAGTCCTAAGGGTAAGTTATCC	125	45.5	57.7
Msph598	*Methanosphaera*	TTTTTTTTTTTTGCTACAAGCTTTCACCAAAG	598	45	57.6
Msph743	*Methanosphaera*	TTTTTTTTTTTTTTACTCACCGTCAAGATCGT	743	45	57.0
Msph673	*Methanosphaera*	TTTTTTTTTTTTCCCCTACCCCGGTAGTAC	673	66.7	58.5
Msph539	*Methanosphaera*	TTTTTTTTTTTTAACAGCTACCACTCGAGCTG	539	55	59.7
Msph841	*Methanosphaera*	TTTTTTTTTTTTGGCACTGGAACAACTCGA	841	55.6	57.8
MsphS193	*Methanosphaera stadtmanae*	TTTTTTTTTTTTCTTAGGCGAAAAATACATTTC	193	33.3	57.5
MsphS838	*Methanosphaera stadtmanae*	TTTTTTTTTTTTGGAACAACTCGAGGCCA	838	58.8	57.5
MsphS174	*Methanosphaera stadtmanae*	TTTTTTTTTTTTCAGGCATAACCTAATATCCAG	174	42.9	57.2
Mspi165	*Methanospirillum*	TTTTTTTTTTTTACGAACTATGGAGTATTACCCC	165	45.5	60.0

Mspi406	*Methanospirillum*	TTTTTTTTTTTTAGCCAGCCTGTGCTGG	406	68.8	58.2
Mspi422	*Methanospirillum*	TTTTTTTTTTTTTGGTGGACAGCCAGCC	422	68.8	58.3
MspiH174	*Methanospirillum hungatei*	TTTTTTTTTTTTAGTCAGCACGAACTATGGAG	174	50	56.7
MspiH193	*Methanospirillum hungatei*	TTTTTTTTTTTTCGGATCTTTCGTTCGCA	193	52.9	59.4
MspiH620	*Methanospirillum hungatei*	TTTTTTTTTTTTCCTGAACGCCCACCG	620	73.3	59.1
Mthe167	*Methanothermobacter*	TTTTTTTTTTTTTCACCTATCCGGGTTTATC	167	47.4	56.6
Mthe190	*Methanothermobacter*	TTTTTTTTTTTTGTGTTTCGGTGAAGAACCA	190	47.4	57.5
Mthe426	*Methanothermobacter*	TTTTTTTTTTTTCACTTCTGAAAAGCCACCC	426	52.6	59.5
Mthe643	*Methanothermobacter*	TTTTTTTTTTTTCCGACCTCGAGTCATGATA	643	52.6	57.4
MtheT193	*Methanothermobacter thermoautotrophicus*	TTTTTTTTTTTTGAAGGTGTTTCGGTGAAGA	193	47.4	56.9
MtheT176	*Methanothermobacter thermoautotrophicus*	TTTTTTTTTTTTAGGCAGCATCACCTATCC	176	55.6	56.4
UncM283	Uncultured methanogen clone 2-10	TTTTTTTTTTTTCTCTCAAGGCCCATACCC	283	61.1	58.9
UncM301	Uncultured methanogen clone 2-10	TTTTTTTTTTTTCCAACTCTGGGCTCCCT	301	64.7	58.6
UncM385	Uncultured methanogen clone 2-10	TTTTTTTTTTTTCTAGGAATTCCCTCATCGG	385	52.6	58.9
UncM544	Uncultured methanogen clone 2-10	TTTTTTTTTTTTATAAAATCGACCACCACTTG	544	40	57.1
UncM869	Uncultured methanogen clone 2-10	TTTTTTTTTTTTTCCCCAAGTAGCAGACTTAA	869	45	57.2
MUncM625	Uncultured methanogen clone 2-10	TTTTTTTTTTTTGTCTCCTCGGAAGACGGA	625	61.1	58.8
MUncM836	Uncultured methanogen clone 2-10	TTTTTTTTTTTTATGCCCCCAAAGGACTT	836	52.9	58.6
Con	Control	AGGAAGGAAGGAAGGAAG			
NbCon	Non-binding control	AGAGAGAGAGAGAGAGAG			