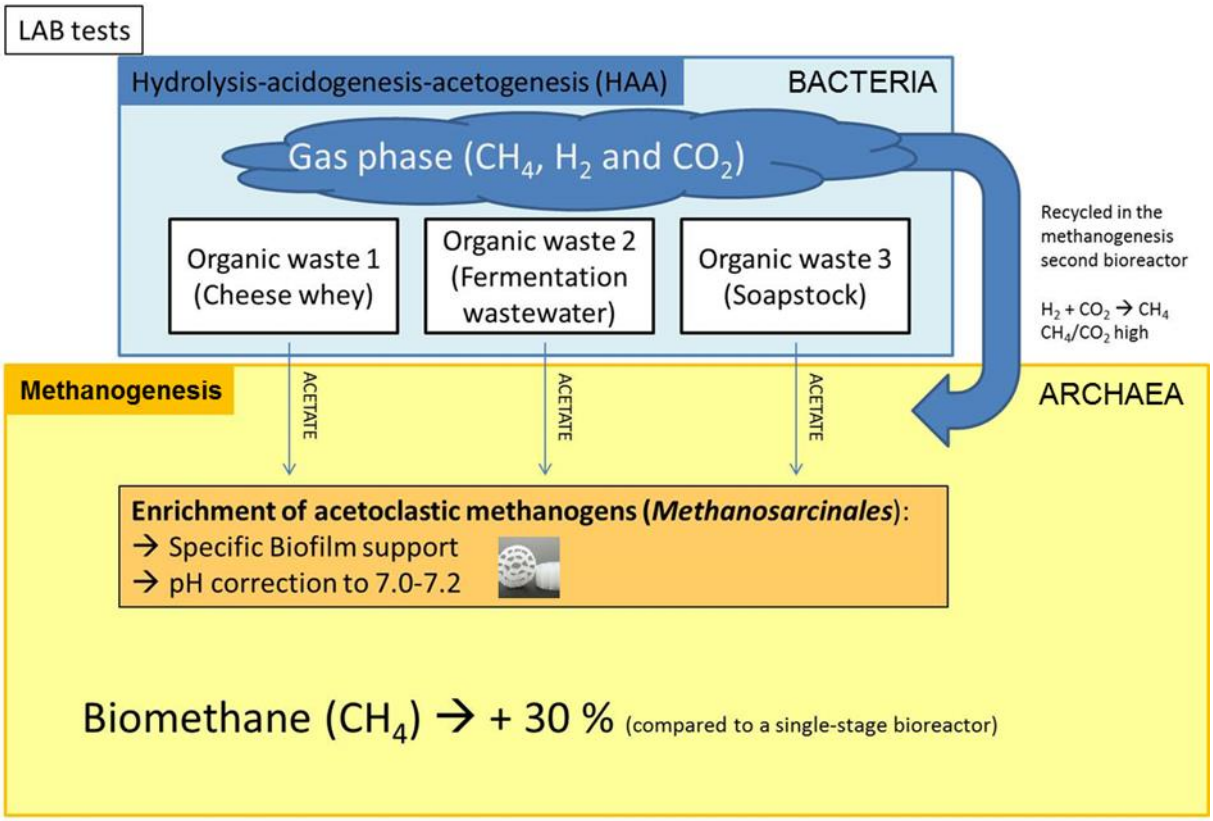
Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Eidgenössisches Departement für Umwelt, Verkehr, Energie und Kommunikation UVEK

Bundesamt für Energie BFE
Sektion Energieforschung

Final report 23.05.17

Two -phase ANaerobic digestion for Aqueous Industrial waStes (TANAIS)



University of Applied Sciences and Arts
of Southern Switzerland

SUPSI

Datum: 23.05.17

Ort: Manno/Bellinzona

Auftraggeberin:
Bundesamt für Energie BFE
Forschungsprogramm Bioenergie
CH-3003 Bern
www.bfe.admin.ch

Auftragnehmer/in:
Scuola universitaria professionale della Svizzera italiana (SUPSI)
Dipartimento tecnologie innovative (DTI)
Mechanical Engineering and Materials Technology Institute (MEMTi)
Galleria 2, Via Cantonale 2c
CH-6928 Manno
www.supsi.ch

Autor/in:
Dr. Pamela Principi, SUPSI – DTI -Mechanical Engineering and Materials Technology, pamela.principi@supsi.ch
Ing. Marco Mattogno, SUPSI – DTI -Mechanical Engineering and Materials Technology, marco.mattogno@supsi.ch
Dr. Nicola Storelli, SUPSI – DACD –Laboratory of Applied Microbiology, nicola.storelli@supsi.ch

BFE-Bereichsleitung: Sandra Hermle, sandra.hermle@bfe.admin.ch
BFE-Programmleitung: Sandra Hermle, sandra.hermle@bfe.admin.ch
BFE-Vertragsnummer: SI/501264-01

Bundesamt für Energie BFE
Mühlestrasse 4, CH-3063 Ittigen; Postadresse: CH-3003 Bern
Tel. +41 58 462 56 11 · Fax +41 58 463 25 00 · contact@bfe.admin.ch · www.bfe.admin.ch

Abstract

The TANAIS project deals with the **exploitation of industrial waste biomasses as renewable energy source** and the optimization of the anaerobic digestion process to increase the methane yield.

The theme of energy from biomass is of foremost importance worldwide and the decarbonisation strategy to support the energy demand is shared -albeit with some differences- at European level.

To increase the potential production of renewable energy, one opportunity is represented by the exploitation of waste from industrial processes through the conversion in biogas by means of anaerobic digestion (AD). To make the AD competitive versus others options, three different aspects could be optimized: the first is the choice of un- or under-exploited biomasses such as the liquid industrial wastes that are relevant to the region in terms of quality and quantity and/or are -up to now- treated with disposal costs; second point is the increase of the methane yield in the process; the third aspect is the recovering from the digested sludge of useful compounds with high added value e.g. phosphates and nitrates.

The present project aims to increase the production of methane assessing the potential for processing three different industrial organic wastes. The three biomasses chosen are whey from cheese production (CW), because it represents one of the largest organic waste flows of the Cantone Ticino, the fermentation wastewater from pharmaceutical processes (FWW), and the soapstocks from food processing companies (SK). Nowadays these wastes of key industrial sector in Ticino are not really valorized but just disposed with extra costs.

To increase the bio-methane yield, the hydrolysis/acidogenesis/acetogenesis (HAA) step of the AD process is physically separated from the final methanogenesis step using an innovative two-stages bioreactor, dimensioned also with microbial kinetic parameters.

At first, the best methane yields has been assessed for each waste in single stage bioreactor to be used as reference. The operational parameters and the maximum methane yield is recorded. Then the three industrial waste have been tested separately with the operational parameters that gave the best performance in the reference tests. A yield greater than 30% for one substrate is deemed satisfactory to reach the milestone set in the project.

The proposed process is innovative respect to the state of the art as a network of reactors are operated to foster ad hoc selected metabolic pathways. In this way, in each bioreactor a specific active microbial community transforms the organic matter with unique kinetics parameters. The sensitive step of the methanogenic phase is enriched by developing biofilm structures to have a stable, resistant and resilient acetoclastic methanogens community, less subject to washout.

Among the three substrates tested, FWW and SK experienced not satisfactory methane yields due to physical chemical characteristics such as sulphur compound presence and difficult solubility. Instead **CW gave the most promising results obtaining a methane yield about two times higher than the reference tests.** The data will have to be validated in a scale up phase in strict collaboration with the industrial partner involved.

Table of Contents

Contents

List of Abbreviations

AD: Anaerobic digestion

HAA: hydrolysis/acidogenesis/acetogenesis phase of the anaerobic digestion

CW: Cheese Whey (LATI SA)

FWW: Fermentation wastewater (Biotech Gnosis SA)

SK: Soapstock (Sofinol SA)

BMP: Bio-methane potential

DGGE: Denaturing Gradient Gel Electrophoresis

FISH: fluorescent *in situ* hybridisationFSC: Forward Scatter

SCC: Side Scatter

CSTR: continuous flow stirred-tank reactor

OLR: organic loading rate

HRT: hydraulic retention time

SRB: sulfate-reducing bacteria

AOM: anaerobic oxidation of methane

SEM: scanning electron microscope

TS: total solids

VS: volatile solids

VFA: volatile fatty acids

COD: chemical oxygen demand

Introduction

Political framework on the use of biomasses as renewable energy source.

In Switzerland, since 2007, the Federal Council has been based its new energy strategy on renewable energy. This has been coming together with an increased sensibility over efficiency in general as well with the plan for the establishment of new power plants for electric power generation. Following Fukushima accident in 2011, the Federal Council and the Parliament decided on Switzerland progressive withdrawal from nuclear energy production: this decision led to the development of the so-called “Energy Strategy 2050”. In 2013 the Federal Council submitted to the Parliament the first set of actions with in the Energy Strategy 2050 plan. A first draft was approved by the Parliament and will be voted by popular referendum on May 2017. In this draft the Council wishes to **significantly develop the existing potential for energy efficiency and exploit the potential of hydro power and the new renewable energies (sun, wind, geothermal, biomass)** and to initiate decommissioning the five nuclear power plants when they reach the end of their service life and not to replace them with new ones. By means of CTI seven **Swiss Competence Centres for Energy Research (SCCERs**) have been funded with the mandate of focus the research on seven different topics: SCCER BIOSWEET (http://www.sccer-biosweet.ch/) focuses on research and implementation of biomass conversion processes with a high level of technological readiness (http://www.bfe.admin.ch/energiestrategie2050/). The TANAIS project is co-funded by the BIOSWEET SCCER program.

In 2007 the EU Commission published the ‘Renewable Energy Road Map — Renewable energies in the 21st century: building a more sustainable future’ (COM(2006) 0848) setting out a long-term strategy for renewable energy within the Union with an outlook till 2020, proposing a **mandatory target of using renewable energy sources to meet 20% of EU energy consumption needs by 2020**. The Commission, in its communication of 6 June 2012 entitled ‘Renewable energy: a major player in the European energy market’ (COM(2012) 0271), identified the areas in which efforts should be stepped up, from now on till 2020, for the EU’s renewable energy production. This document also highlights the pathway to continue increasing renewable energy production with a foresight to 2030 and beyond: specifically for **renewable energy technologies to become less costly, more competitive and, ultimately, market-driven.**

The anaerobic digestion as process to convert waste biomass in energy.

Although the production of biogas through anaerobic digestion (AD) is not new, commercial AD processes are often operated well below their optimal performance and this is due to a wide variety of factors. Most existing full-scale plants are designed in a single-stage configuration for economic reasons. The microbial community in the AD process comprises different kind of specialized microorganisms, such as hydrolytic-fermentative bacteria, acetogenic bacteria and methanogenic archaea. In general, methanogens have slower growth rates compared to others microorganisms involved in the AD and are more sensitive to environmental stress (Demirel & Scherer 2008; Karakashev et al. 2005; Shah & Mahmood 2014; Wijekoon et al. 2011). The spatial separation of the

first steps from the methanogenesis process in a two-stages bioreactor has been proven to be beneficial in terms of process efficiency (biogas production and velocity) and stability (Blonskaja et al. 2003; Ghosh et al. 2000; Sieger 2001; Liu et al. 2006). Optimizing the reactions separately in different bioreactors may lead to a larger overall reaction rate and methane yield. Accordingly, it is possible to operate the acidification phase at low pH such as 4.0 – 5.0, which facilitate the developing of specific microorganisms producing acetate used then for methane production (pH 6.8 - 7.5). Moreover a multi-stage system requires less digester volume to handle the same amount of input volume because it has lower hydraulic retention times (HRT) and allows higher organic loading rates (OLR) than a single-stage system. With the two-stages occurring in distinct bioreactors, it would be theoretically possible to increase the rate of methanogenesis that is normally the limiting step of the AD process. However, a disadvantage of multi-stage anaerobic digestion systems is an higher operation and maintenance costs compared to a single-stage systems (Office of Water 2006).

Literature reference for increased methane yield in a two-phase configuration.

Literature offers evidence regarding the use of two-stages bioreactors fed with different feedstock and resulting in an improvement of methane production compared to the one-stage bioreactor. For example, methane production in the second stage from food waste was significantly improved by the hydrogen production by a factor of more than 2-folds compared to a single-stage bioreactor. The maximal methane production resulted approximately 0.5m^3CH4 per kg of volatile solids (VS), which corresponds to an increase of ca. 2.5 KWh/kgVS for the two-stages bioreactor compared to a common one-stage bioreactor using food waste as feedstock (Zhu et al. 2011). Similar methane increase yields using cheese whey in two-stages bioreactor were shown also in others studies (Saddoud et al. 2007; Bertin et al. 2013). All these studies showed a maximal production of approximately 0.25m^3CH4/kgVS to 0.30 m^3CH4/kgCOD corresponding to a hypothetical energy production of ca. 2.5 – 3.3 KWh every kg of VS or COD of cheese whey, with an increase of 1.0 -2.0 KWh/kgVS or COD compared to a single stage bioreactor.

A two-stage hydrogen-methane process from household solid waste has been successfully demonstrated: methane production was 21% higher (0.5m^3CH4/kgVS) than in the one-stage process with an estimated theoretical energy production increase of approximately 1.0 KWh every kg of VS of feedstock (Liu et al. 2006). Similar results were also found in the disposal of potato wastes, where the two-stages bioreactor always produced more methane compared to the one-stage bioreactor (Kraemer & Bagley 2007; Ueno et al. 2007; Cooney et al. 2007; Zhu et al. 2008).

Aim of the project

Main goal of TANAIS project was to develop and realize a two-stages bioreactor and to assess its competitiveness in term of methane production compared to the common single-stage bioreactor for the industrial sector involved in the project.

To reach this goal, different communities of microorganisms involved in the AD (HAA and methanogens) were enriched and specialized for each specific substrate. Indeed, most studies regarding two-stages bioreactor focus on process optimization by means of an “engineering approach”, hence aiming at determining optimum environmental and operational parameters. In this framework, TANAIS was run to specifically characterize and optimize different microbial communities which play a key role in the AD process.

In detail TANAIS aimed at:

- evaluating the potentiality and the limits of the AD process, for the three different substrates investigating physical, chemical and microbiological limits of the process in a suspended biomass and single stage bioreactor. The methane productions on the relative OLR conditions were the basis point to evaluate the potential increase by the two-stage bioreactor. Three chosen substrates were deemed important for Ticino economy and were namely: cheese whey, pharmaceutical fermentation wastewaters and soapstocks from oil processing industry.

- optimizing the hydrolysis/acidogenesis/acetogenesis (HAA) for critical organic wastes relevant to the Ticino region. Growing conditions such as pH, temperature, stirring and extra nutriments (vitamins and trace elements) are optimized together with OLR and HRT in order to improve microbial community for each organic waste, to finally reach higher acetate yield. The acetate can be then converted into bio-methane in the second methanogenesis bioreactor.

- optimizing the methanogenesis process, by laboratory enrichment of an optimal community of acetoclastic methanogens. The optimal methanogenic community was grown using specific biofilm supports that sustain a more resistant and resilient community.

- increasing methane production for a given substrate, at lab scale, at least 30 % with respect to a conventional one-stage digester. This cutoff value was chosen as go/no-go factor for the process second phase implementation: the scale-up up to a pilot test configuration, to be installed on the site of an interested industrial partner.

Material and Methods

Project activities were planned in a 18 months' timeframe. The competences of the two partners - namely the Bio Environmental Technologies (BET) team at MEMTI (former ICIMSI) and the Laboratory of Applied Microbiology (LAM), both at SUPSI, complemented each other covering two key project needs: process engineering with the setting of 1-stage and 2-stages bioreactors (at lab scale) by BET, and microorganisms optimization, enrichment and characterization by LAM.

Chemical and physical analysis. Standard chemical physical characterization was performed routinely for each substrate tested to allow data normalization on organic matter and comparison of different substrates. Total solids (TS), volatile solids (VS) and ash percentages (w/w) have been measured according to standard methods (APHA, 2005). The biogas composition has been measured by GA5000 gas analyzer (Geotech). Chemical oxygen demand (COD), acetate, volatile fatty acids (VFA), alkalinity, sulfide (H_2S) and pH have been measured after centrifugation (5000 rpm during 10 min) and filtration of the supernatant using 0.45 µm specific filtration unit. Specific kits were provided by Merck AG (Zug, CH), Hach (Rheineck, CH) and r-Biopharm (Darmstatd, Germany) and have been used to evaluate the chemical compounds according to the manufacturer's instructions.

Bio methane potential assays (BMP). This test allows to quantify the potential biogas and methane production in standard conditions per organic matter measured as volatile solids. It is also useful to evaluate the occurrence of toxicity effect on methanogenesis. BMP experiments have been carried out in triplicates at 37 °C in 1.050L sealed bottles, according to Owen et al. (Owen et al.

1979). Tests started-up by adding 270 mL of inoculum (digested sludge from IDA Gordola) and 30 mL of substrate (CW, FWW or SK), corresponding to a 10% dilution factor, and further incubated at 37 °C. Monitoring has been carried out until pressure inside the bottles reached stable conditions (up to 40 days). Methane content has been estimated measuring pressure increase inside sealed bottles, applying ideal gas law equation (PV = nRT) and assuming a methane content of 60%. Blank experiments with water and sole inoculum have been also carried out, in order to evaluate background gas productivity of the inoculum, to be used as baseline value. The content of the sealed bottles has been flushed with nitrogen (N_2) in order to secure anaerobic conditions.

Carriers for specific biofilm support. Attached-growth systems are advantageous with respect to suspended ones as they limit the loss of biomass that can be an important issue for slow-growing microorganisms as methanogens. Shape, functional area and material composition are just some of key variable affecting biofilm adhesion and growing. Different supports have been evaluated to best grow a strong microbial community in the methanogenenic phase. The ”white carrier” (AnoxTM K5) produced by Veolia (Paris France), is a disk-shaped carrier with a nominal diameter of 25 mm for biofilm support growth in biological wastewater treatment. Used media are produced by extrusion machines, have a protected area of 800 m^2/m^3, a specific weight of 118 kg/m^3, with a unit number per m^3 of 331.000 units/m^3. The “black carrier”, (HX17KLL) hel-x biocarriers®, produced by Christian Stöhr GmbH & Co.KG (Marktrodach, Germany), is a cylindrical medium made of HDPE recycled, with a diameter of 17mm and length 17mm. The total specific surface is 602 m^2/m^3 with a protected surface area for biofilm growth of 393 m^2/m^3. The weight is 125 kg/m^3 and units number per m^3 is 153.000 units/m^3.

Microorganisms growing conditions. The experiment of acetate production optimization has been carried out with 150 ml sealed bottles filled in with 20 ml of inoculum (digested sludge from the digester in Consorzio Depurazione Verbano CDV, Gordola TI CH) in an anaerobic atmosphere composed of 20% CO_2 and 80% N_2. The cultures have been kept at 37°C and shacked at 150 rpm. During early stages of run-up, fixed volumes of 1.5 ml and 2.0 ml have been added, and removed two times per week (HRT = 40 days; 0.5 ml per day), respectively. Each organic waste (CW, FWW or SK) has been added starting from low COD concentrations, to provide adequate and sufficient time to the communities to specialize to each substrate. The concentration of acetate, pH and the number of cells has been measured once per week. The acetoclastic methanogens enrichment has been carried out with 600 ml sealed bottles, filled with 300 ml of inoculum (digested sludge from digester in Consorzio Depurazione Verbano CDV, Gordola TI CH) in an anaerobic atmosphere composed of 20% CO_2 and 80% N_2. Cultures have been kept at 37°C and shacked at 150 rpm. During early stages of run-up, fixed volumes of 6.0 ml and 8.0 ml have been added, and removed two times per week (HRT= 150 days; 2.0 ml/day), respectively. Acetate has been added in a low OLR of 0.203 g/ld for safety reason. Acetate concentration, pH and number of cells has been measured once per week.

After one month, a sample of enriched acetoclastic methanogens sludge (300 ml) has been split in 3 different sealed bottles of 300 ml capacity (100 ml liquid sample), the first with only suspended biomass (control), the second with “white biofilm carriers” and the third with “black biofilm carriers”. During early stages of this set-up, fixed volumes of 6.0 ml and 8.0 ml have been added, and removed, on Mondays and Thursdays (HRT= 150 days; 2.0 ml/day), respectively. Acetate has been added in a low OLR of 0.203 g/ld for safety reason. Acetate concentration, pH and number of cells has been measured once per week. The liquid sample was fixed with 4% formaldehyde (w/v final concentration). The carrier sample was PBS-washed (Phosphate Buffer Saline 1x, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) by shake and sonication (5x 10 sec 10% power) and then fixed with 4% formaldehyde solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland).

Flow cytometry. The coenzyme F420 involved in methanogenesis causes an intense auto-fluorescence of cells under excitation by shortwave UV light (max Abs 420nm). This auto-fluorescence is a diagnostic feature and can be used to check cultures of methanogens by flow cytometry. BD Accuri C6 cytometer (Bect Dickinson, San José, CA, USA) equipped with two lasers (488 nm, 680 nm), two scatter detectors, and four fluorescence detectors (laser 488nm: FL1 = 533/30, FL2 = 585/40, FL3 = 670; laser 640 nm: FL4 = 670) has been used for this purpose. Flow cytometer parameters have been used for event characterization: forward scatter (FSC), which is often correlated to particle size, and 90° light scatter (SSC), which is considered to be related to the size and internal granularity of the particles. Thresholds have been applied first to forward scatter (FSC-H 10'000), for the exclusion of debris and abiotic particles, and subsequently to the FL1 filter for the detection of natural fluorescence of coenzyme F420.

Denaturing Gradient Gel Electrophoresis (DGGE). DGGE is a useful technique commonly applied for monitoring changes in microbial communities. Total microbial community DNA is extracted and, by amplifying a fragment specific for a taxum with specific primers, the analysis is focused on the changes occurring in that taxum. The amplification product is then separated by gel electrophoresis that results in a band pattern for each sample (a sort of fingerprint of the analyzed microbial community): each band indeed corresponds to an operational taxonomic unit (OTU) and the number of bands are an indication of the biodiversity in the sample. The cells have been lysed by a bead-beating procedure, followed by a extraction using the kit "Fast DNA Spin Kit for Soil" (MP Biomedicals, LLC Santana California,USA) according to the manufacturer's instructions. Bacterial and archaeal specific 16S rDNA have been obtained by PCR amplifications using the primer combination 338-f-(GCTGCCTCCCGTAGGAGT) 907GC-r-(CCGTCAATTCCTTTRAGTTT) and ARCH344GC-f-(ACGGGGYGCAGCAGGCGCGA) - ARCH915-r (GTGCTCCCCCGCCAATTCCT) respectively. A GC-clamp of 40 nucleotides has been hanged at the 3'end of primer 907GC-r and ARCH344GC-f (Muyzer 1993). The length of the product was approximately 600bp. Amplifications have been performed using a touchdown PCR protocol with 10 cycles at temperatures ranging from 65 to 56 °C, and by reducing the annealing temperature for 1°C every cycle (Bottinelli 2008). This procedure has been followed by 20 additional cycles at an annealing temperature of 55°C. After the initial denaturation step at 94 °C for 5 min, denaturation, annealing and polymerization steps were 1 min, 30 sec, and 1 min long, respectively. PCR amplification products have been then separated by DGGE on a DCodeTM Universal Mutation Detection System (Bio-Rad Laboratories, Cressier, Switzerland) during 5h at a constant voltage of 150 V and at 60 °C in a 30 to 60 % vertical denaturing gradient, where 100% denaturant was 40% formamide and 7 M Urea. Gels with banding profiles have been photographed with a Quantum ST4 System (Vilber Lourmat, Eberhardzell, Germany) using an UV transilluminator after staining with a GelRed (Biotium, Hayward, CA, USA) for 25 min. Images have been then analyzed by the free software ImageJ (https://imagej.nih.gov/ij/).

Fluorescent in situ hybridisation (FISH). The FISH technique allows visualizing and identifying microorganisms under microscope. Cells are made permeable to probes keeping the spatial conformation inside the sample. Using taxa-specific probes, labeled with fluorochromes, it is possible to visualize and count target populations in microbial communities. Acetoclastic methanogens from the family of *Methanosarcinaceae* in the enrichment experiments have been identified and counted with species-specific Cy3-labeled oligonucleotides MSMX860 (Raskin et al. 1994) with 2 and 5 µl aliquots of paraformaldehyde-fixed samples (n = 3) spotted onto gelatin-coated slides [0.1% gelatin, 0.01% $KCr(SO_4)^2$] (Glöckner et al. 1996). Hybridizations have been performed as described in previous studies (Moter and Göbel, 2000). Slides have been treated with Citifluor AF1 (Citifluor Ltd.,

London, UK) and examined by epifluorescence microscopy using filter sets F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, and D460/50 for DAPI) and F41 (AHF Analysentechnik; HQ535/50, Q565LP, and HQ610/75 for Cy3). Microorganisms have been counted at a 1,000-fold magnification in 40 fields of 0.01 mm^2 each.

Scanning Electron Microscopy (SEM). To study the spatial interaction between carriers and microorganisms, electron microscopy has been applied. Biofilm samples on carrier chips have been observed by SEM. To observe biological samples containing water, a fixation step is needed to maintain structural integrity under vacuum conditions: usually a step is performed with paraformaldehyde or glutaraldehyde. The main drawback of this procedure, besides the time consuming protocol, is the alteration of the specimen structure due to the chemical bonding of the fixative (polymerization). Recently a new protocol has been published (Takahashi et al. 2015) using an ionic salt (IL) liquid at room temperature and with low vapor pressure. Samples have been dehydrated with an ethanol series and incubated for 30 minutes at room temperature with the IL solution ([BMIM][BF_4]) following the cited protocol (Takahashi et al. 2015). To avoid electron loading of the samples, the surface has been sputtered with a gold layer. Samples have been observed with the JEOL 6010-LA SEM (Jeol Italia spa, Basiglio (MI) Italy) equipped with an EDX sensor. Observations have been carried out in Secondary Electrons mode (SEI) and Backscattered Electrons mode (BES) in low vacuum mode, with a voltage of 10.0 kV with a spot size of 30 and 60 µm.

Results and discussion

Test substrates: selection and characterization.

Three different organic wastes, as said relevant to the Canton Ticino economic environment and community, were selected to be evaluated for their potentiality in biogas production:

1. **Acid cheese whey** (CW) provided by LATI SA, the larger cheese producer in Ticino. LATI has a daily production of acid CW of about 20'000 liters, but only 5'000 l are used for feeding pigs and the remaining 15'000 liters are waste with a high industrial disposal cost.

2. **Fermentation wastewater (FWW)** provided by Biotech Gnosis SA, a biotechnology company specialized in manufacturing and sales of fermentation raw materials and natural finished products used in pharmaceutical, nutraceutical, cosmetic, veterinary, and agricultural industries. Due to the good stability of the FWW, Biotech Gnosis stored a 100 liters barrel full of feedstock for this project.

3. **Soapstock (SK)** provided by Sofinol SA, a Company extracting, refining and mixing specialty oils coming from fish. They have approximately a monthly production of SK.

All selected substrates were characterized in the BET laboratory, as shown in Figure 1 below. These three substrates resulted to be very different in terms of organic matter content and pH, in addition the first two (CW and FWW) are liquid at room temperature, while the third (SK) is solid.

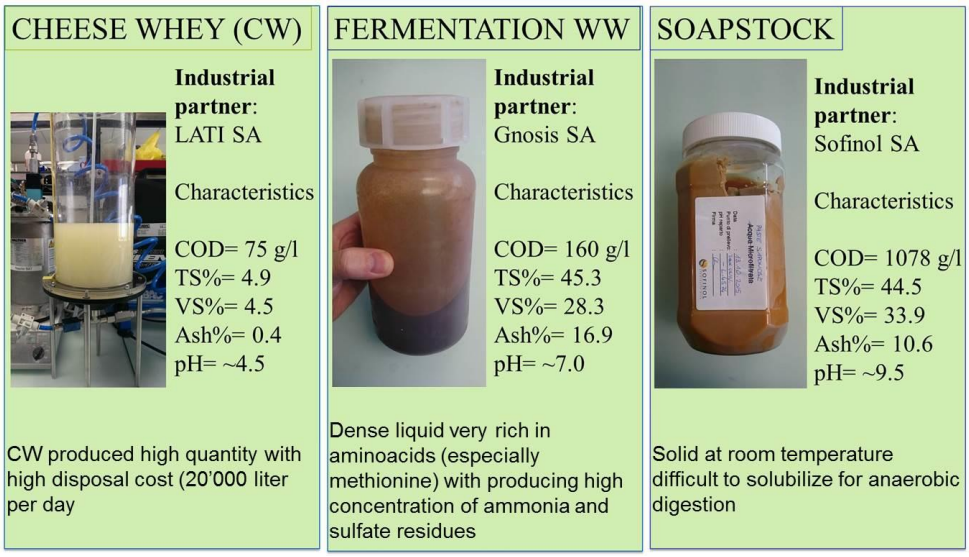


Figure 1. Characterization of all the selected organic wastes.

All three substrate were also characterized in terms of BioMethane Potential (BMP). The first two organic wastes (CW and FWW) showed a classical profile of methane production with a sigmoid curve: a short lag-phase to acclimate the microbial community to the different test conditions and an exponential methane production, being higher for FWW and lower for CW; the last phase is a plateau due to the reaching of the steady methane production. The third feedstock (SK) had a trend lower than even the control (blank digestate) evidencing the main problem of low solubility at room temperature responsible for a difficult substrate access by microorganisms: indeed, once the SK had been diluted 1:50 with water, the resulting BMP graph was similar to CW and FWW (data not shown).

Single phase reactors tests.

For each substrate a reference value of methane production in "standard" CSTR process had to be obtained for referencing purposes: to obtain a valid reference value, microorganisms acclimatation to substrates had to be taken into account. For this reason, small scale reactors were set up (data shown in annex 1). Only after reaching stable methane production (*i.e.* around 30 days, please see annex 1), microorganisms could have been used as inoculum to evaluate the best OLRs in a second set of tests (also called "optimization phase"). The starting OLR (~0,3-0,4 kgCOD/m^3d) has been chosen as starting point as it is typical for Continuous-flow Stirred-Tank Reactor (alias CSTR) reactors.

The optimization phase (HAA hydrolysis acidogenesis, acetogenesis) was aimed at maximizing acetate production.

Cheese Whey (CW). After start-up time (see Annex 1), a second optimization phase (Figure 2), was started. During optimization, CW dilution rate has been gradually reduced, increasing the OLR, up to inhibition of the process. CW was daily added in a single amount and once a week re-added but in a 3 fold amount.

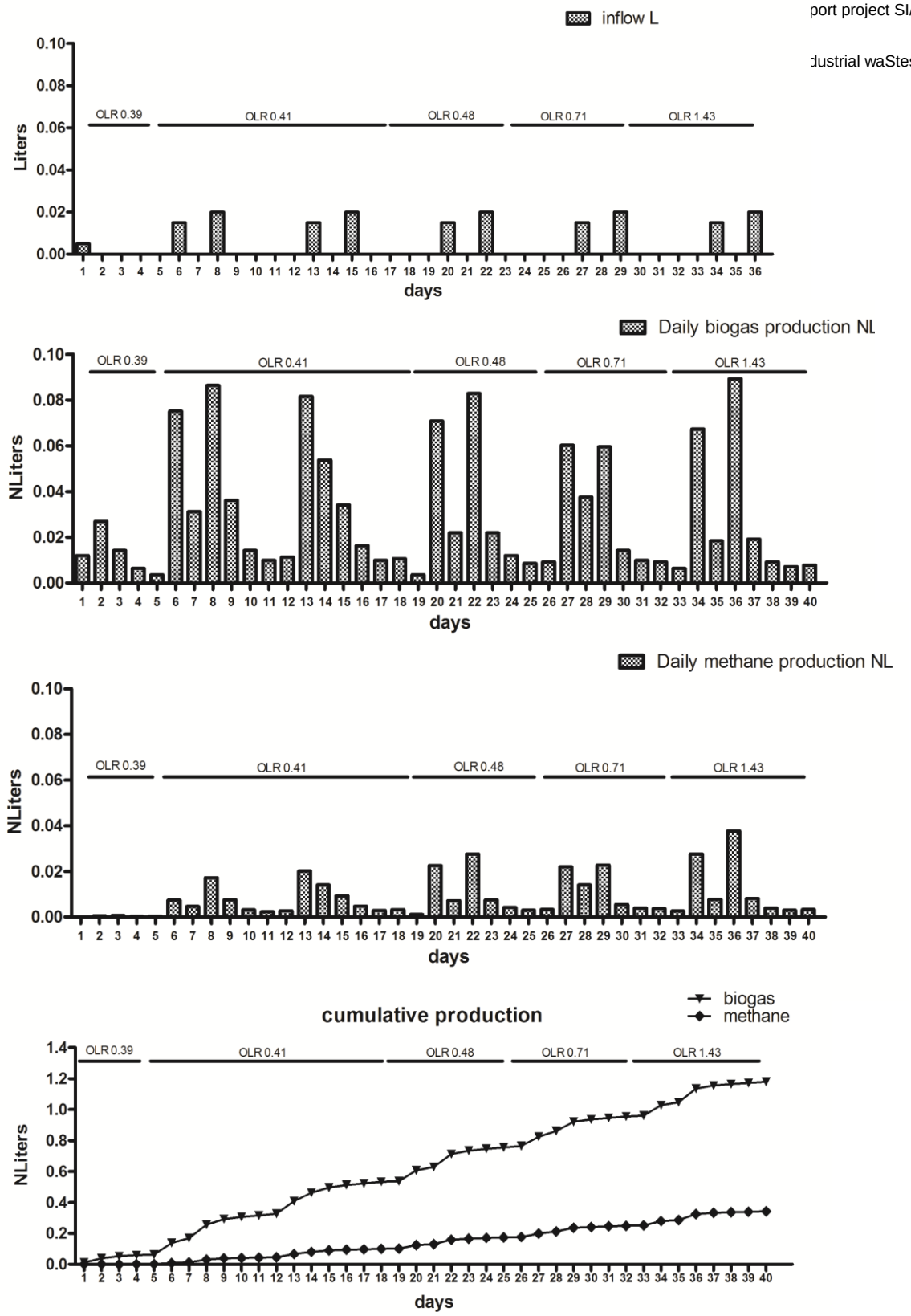


Figure 2. Optimization of an one-stage bioreactor fed by CW. From top to bottom: feeding of CW, biogas and methane daily and cumulative production in the bioreactor. The lines indicate the corresponding OLRs increase. Best results in methane yield were reached with an OLR of 0,39 kgCOD/m^{3}d. Increasing the quantity of CW fed into reactors and the OLR resulted in a higher biogas quantity and methane production, but a decrease in the yields has been observed for all the parameters expressed (CW, COD and VS).

The methane production reported for the different organic load is summarized in Table 1: it is expressed as Nm^3/Ton VS (table 1, far right column) but it has to be underlined that it showed some uncertainty (results are indeed marked with an asterisk) due to a non-optimal sample processing. These were lab-scale run experiments and used process and procedure need to be further improved with a step for fat homogenization.

Table 1. Methane production for different OLR of CW.

	Methane production		
OLR $\frac{Kg\ COD}{m^3\ d}$	$\frac{Nm^3}{m^3 CW}$	$\frac{Nm^3}{Ton\ COD}$	$\frac{Nm^3}{Ton\ VS}$
0,39	10,9 ± 0,9	175,8 ± 15,3	242,3 ± 20,8*
0,41	9,3 ± 0,4	164,3 ± 7,6	322,7 ± 15,1*
0,48	8,3 ± 0,6	146,8 ± 10,5	288,3 ± 20,7*
0,71	8,7 ± 0,4	153,4 ± 7,5	301,4 ± 14,7*
1,43	6,2 ± 1,4	108,9 ± 24,9	213,9 ± 48,9*

*these values are affected by heterogeneity of the CW as fats have no uniform distribution.

The whole process of degradation showed evident signs of inhibition at OLR around 0.41-0.48 kgCOD/m^3d; indeed a gradual increase of the COD content in the outflow and an increase of volatile fatty acids (VFA) in the sample were observed, together with a lack of a system buffer capacity (alkalinity). This was evident from pH acidification at 1.43 OLR (Table 2). The main problem of the CW's degradation refers to the inverse relationship between alkalinity and OLR.

Using CSTR in combination with suspended biomass, at OLR 0.41-0.48 kgCOD/m^3d, the degradation process loses stability and decreases the percentage of methane yield in the biogas from the peak values of 52%.

Table 2. Key parameters during the optimization of one-stage bioreactor fed by CW.

OLR $\frac{Kg\ COD/}{m^3\ d}$	pH	Alkalinity mg/L	VFA mg/L	outflow COD mg/L
0,39	7,6-7,4	803 ± 145	0	374 ± 113
0,41	7,6-7,4	870 ± 35	0	344 ± 66
0,48	7,4-7,2	-	-	-
0,71	6,8-6,4	815 ± 48	31 ± 10	382 ± 138
1,43	6,7-6,3	333 ± 23	284 ± 189	1224

Fermentation WasteWaters (FWW). After the start-up period (Annex 1) a second phase, called optimization, was started (Figure 3). During optimization period the FWW dilution rate has been gradually reduced to 1:7, hence increasing the OLR from 0.40 to 0.57 kgCOD/m^3d. This point was proven to be the limit for FWWs to both biodegradability and biogas production. Substrate FWW has been daily added and once a week in a 3 fold amount.

The methane production reported for the different OLR is showed in the Table3.

Table 3. Methane production for different OLR of FWW.

	Methane production		
OLR $\frac{Kg\ COD}{m^3\ d}$	$\frac{Nm^3}{m^3 FWW}$	$\frac{Nm^3}{Ton\ COD}$	$\frac{Nm^3}{Ton\ VS}$
0,40	43,9 ± 6,2	274,6 ± 39,2	155,1 ± 22,1
0,57	25,2 ± 7,1	157,6 ± 44,5	89,0 ± 25,1

FWW showed a high concentration of nutrients and high specific biogas production; the best performance in terms of specific methane production was obtained with an OLR of 0.40 kgCOD/m^3d; increasing feeding rate, biogas production became highly uneven, hence reducing specific methane overall production.

Chemical analysis for the different range of OLRs showed a discontinuous biogas production, that was also affected by an alkalinity and pH decrease, with an increase in the COD output (Table 4).

Table 4. Key parameters during the optimization of one-stage bioreactor fed by FWW.

OLR $\frac{Kg\ COD}{m^3\ d}$	pH	Alkalinity mg/l	VFA mg/l	Outflow COD mg/l
0,40	7,-7,3	1872 ± 48	5835 ± 1347	4730 ± 35
0,57	6,8-7,0	1734 ± 76	5532 ± 520	6862 ± 2345

The process was also characterized by a high level of H_2S yield found in the produced biogas, as much as more than 600ppm in the gas phase. H_2S presence, at higher concentrations, is known to inhibit the microbial activity, particularly the methanogens. Toxic concentrations of hydrogen sulfide increase the instability of the degradation process and makes the biogas production discontinuous and variable (Annex 1). Specific pretreatment could be applied to overcome the inhibition issue. Although the evaluation of substrate specific pretreatments is beyond the scope of the present project, some options have been preliminary evaluated, see Annex 2 for further details.

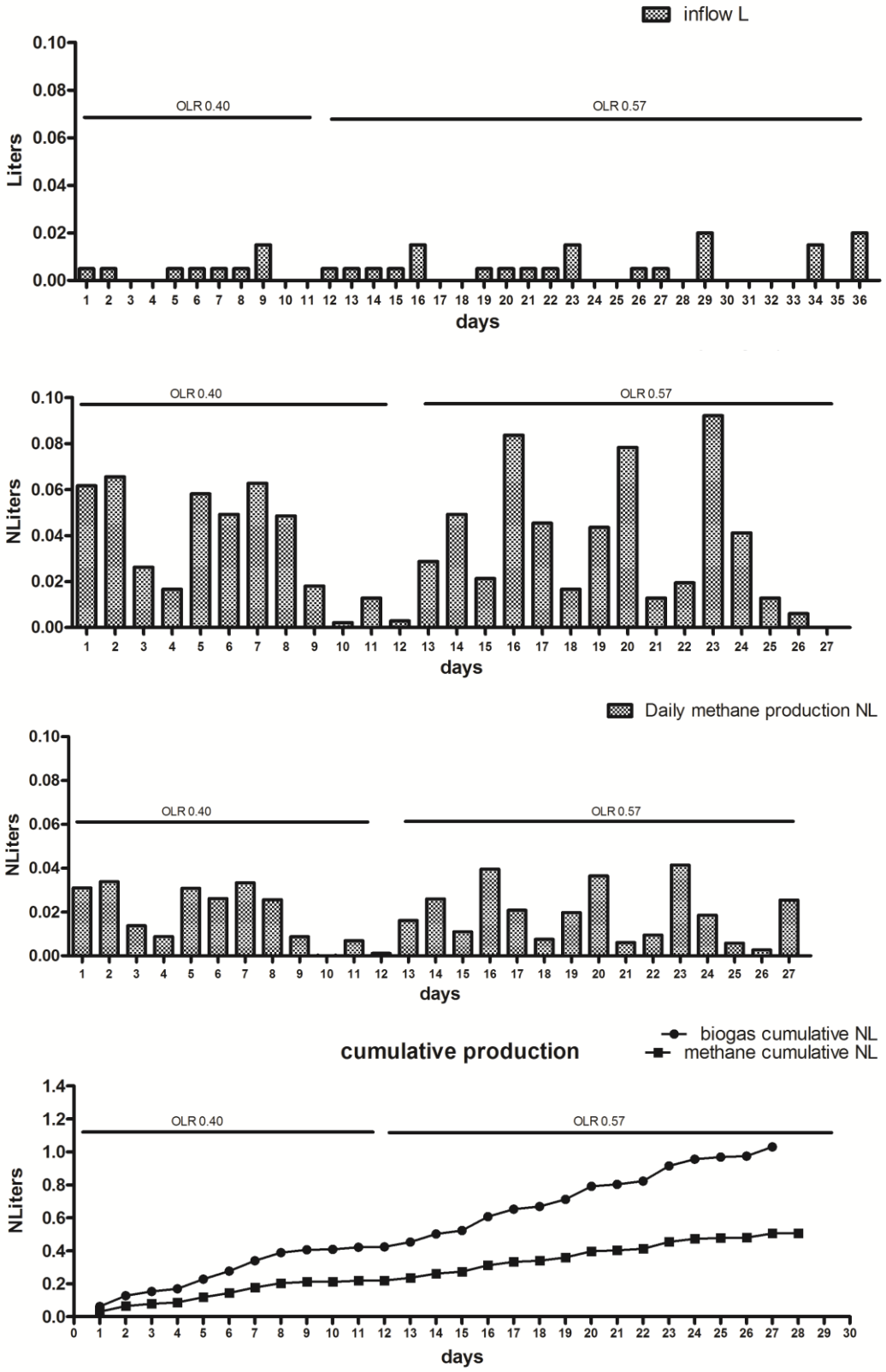


Figure 3. Optimization of an one-stage bioreactor fed by FWW. From top to bottom: feeding of FWW, biogas and methane daily and cumulative production in the bioreactor.

Soapstocks SK. After the startup phase (Annex 1), an optimization step was set-up and kept running for about 3 weeks, where SK was added daily and once a week in a 3 fold amount, hence reducing the dilution rate to increase the OLR. Figure 4 shows the results obtained in terms of biogas and methane daily and cumulative productions during optimization phase. Due to the solid state of SK at room temperature, and the extremely high COD content of the SK, the optimization phase was started with a low OLR, to increase SK solubility. OLR has been gradually increased, until a reduction of methane production activity was recorded (Table 5). On the other hand, considering the COD content in the outflow (Table 6) the data do not show a decrease in the degradation of organic matter; that could instead be converted to CO_2 instead of methane. The test was stopped as a decrease of the specific yields in biogas production referred to the amount of COD, VS and SK introduced was recorded.

Table 5. Methane production for different OLR of SK.

	Methane production		
OLR $\frac{Kg\ COD}{m^3\ d}$	$\frac{Nm^3}{m^3 SK}$	$\frac{Nm^3}{Ton\ COD}$	$\frac{Nm^3}{Ton\ VS}$
0,27	152,4 ± 21,9	140,8 ± 20,2	449,5 ± 64,7
0,39	88,9 ± 16,4	82,1 ± 15,2	262,1 ± 48,5
0,54	81,3 ± 33,8	75,1 ± 31,2	239,9 ± 256

These results can be explained by the low solubility of the substrate that did not present the optimal conditions to be biologically attacked by the active suspended biomass in a CSTR.
The COD measures might have been afflicted by a systematic error due to the low solubility of the substrate; indeed, as shown in “Material and Methods - Chemical and physical analysis”, the soluble COD has been measured after centrifugation and filtration, so the total COD could have not been evaluated in the sample due to the spinning down of the insoluble particles after the centrifugation (that could have also been stuck onto the filter too).

Table 6. Key parameters during the optimization of one-stage bioreactor fed by SK.

OLR $\frac{Kg\ COD}{m^3\ d}$	pH	Alkalinity mg/l	VFA mg/l	outflow COD mg/l
0,27	7,7-7,4	1561 ± 123	0	548 ± 309
0,39	7,9-7,7	1268 ± 0	13	526 ± 56
0,54	7,9-7,3	1092 ± 124	0	445 ± 21

Probably the accumulation of the insoluble substance has been responsible for the decrease performances in methane production as there would not be a lack of dissolved organic material for the microorganisms responsible for the AD process.

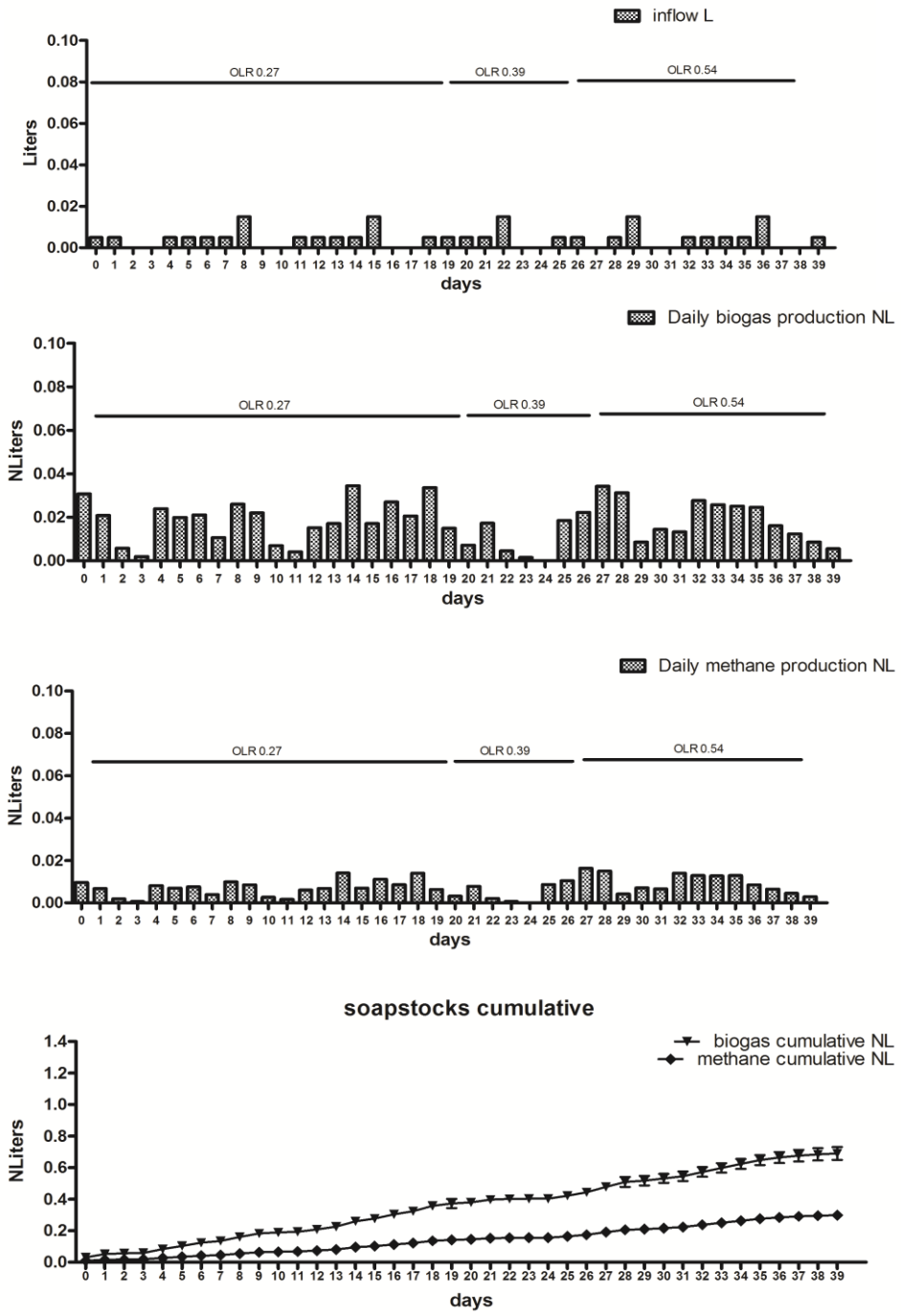


Figure 4. Optimization one-stage bioreactor fed by SK. From top to bottom: feeding of SK, biogas and methane daily and cumulative production in the bioreactor.

Monitoring and optimizing acetate production rates and operation parameters

One of main aims of the project was the optimization of acetate production to increase the performances of the two-stage bioreactor. To achieve this goal, different key microbial growing parameters such as pH, temperature and OLR, have been optimized according to the microbial community that was selected independently for each organic waste, to finally reach the maximal acetate yield. Each substrate was added to an independent HAA bioreactor with microbial communities specifically selected for each chosen organic waste. This specialized bacterial community was set to improve the hydrolysis and the acidogenesis steps of the AD process. The acetate was then converted into methane in the second methanogenesis bioreactor by enriched acetoclastic methanogens.

CW: Cheese whey is a liquid substrate rich in lactose (30 g/l) and proteins (1.5 g/l) with an acid pH around 4.0 and a COD of approximately 60-70 g/l. The dilution rate, at the beginning of the experiment (cheese whey and inoculum), provided a starting pH value of 7.0 and a daily COD of 0.031 g (OLR = 1.55 kgCOD/m^3d) until the concentration of acetate started to increase after about 2 weeks of incubation. At this point the daily OLR of CW has been regularly increased until the production of acetate showed a sudden inhibition at a COD of 0.135 g/d (OLR = 6.75 kgCOD/m^3d), (see figure 5A - black dot and black square). Usually specialization process in heterogeneous anaerobic microorganisms community is slow (Whitman, *et al*., 2006); for this reason the total cells number counted by FC increased about 10 times after approximately 2-3 weeks (figure 5B, grey bars).

Flow cytometry plots of cellular complexity (SSC) and size (FSC) displayed the specialization of the microbial community during the experiment: larger cells have higher values in the y-axis, while cells with higher signal refraction are represented along the x-axis. Observing the microbial community plots from the beginning to the end of the experiment it is possible to see that the different cell signal composition changed increasing its sharpness (figure 5B, bottom). During the first week pH dropped to 5.0 due to CW acidity, allowing a positive selection for acetogenic bacteria (Drake et al. 2008): in this condition, acetogenic bacteria increased in number and in productivity of acetate, hence decreasing the pH to 3.5.

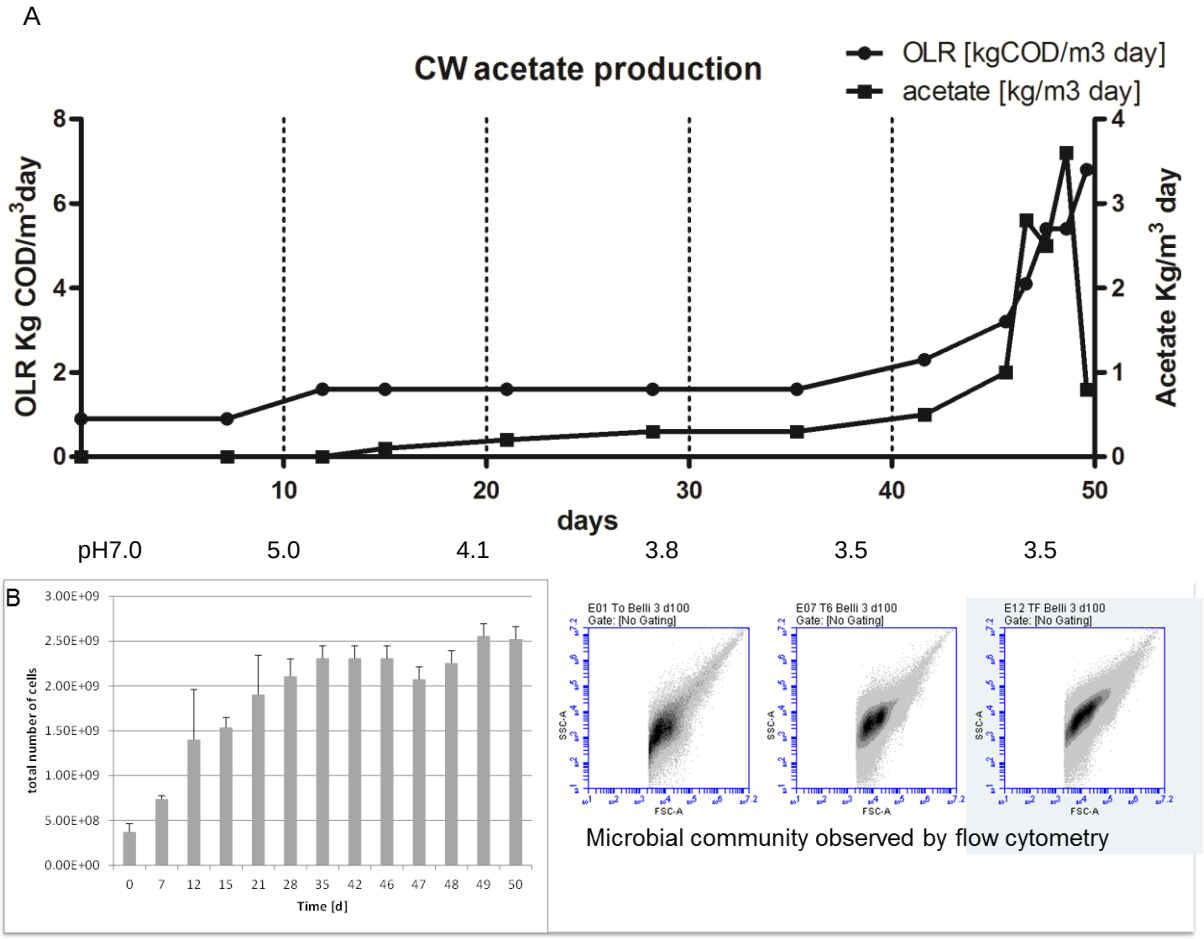


Figure 5. CW optimal acetate production. A) concentration of acetate (black square, kg/d) and OLR (black dot, kgCOD/m3d) in function of the time (days). At the bottom of the figure pH value is reported. B) Flow cytometry results, the left graph showes the total number of cells (grey bars) along the experiment, and the right part showes a plot of the cellular complexity (SSC) and size (FSC) of the microbial community at the beginning, in the middle and at the end of the analysis.

This experiment showed that in laboratory conditions, with small volumes (20 ml), the maximum acetate production for CW is 0.658 g of acetate every g of COD (OLR = 5.403 kgCOD/m^3 d).

FWW: Fermentation wastewater is a viscous substrate, very rich in aminoacids, especially methionine with the S-methyl thioether side chain, with an acid pH around 5.0 and a COD of approximately 200 g/l. The enrichment tests were set to start with a daily COD concentration of 0.010 g/d (OLR = 0.5 kgCOD/m^3d). After about 2 weeks an increase of acetate concentration was measured. The daily concentration of FWW was regularly increased until the production of acetate stopped at 0.051 g/d (OLR = 1.939 kgCOD/m^3d) (figure 6A). Similar to what observed in CW acetate optimization experiment, pH rapidly dropped also in this experiment to an acid value of 5.0, which supports the growth of acetogens responsible for the acetate production. The maximal production of acetate was recorded simultaneous maximal cells number counted by FC (figure 6A and B).

The maximal number of cells was almost doubled compared to CW value, and this might have been due to FWW richness in aminoacids and sugars. Moreover, FC plots of cellular complexity (SSC) and size (FSC) displayed a specialization of the microbial community, also compared to CW results (figure 6B, bottom).

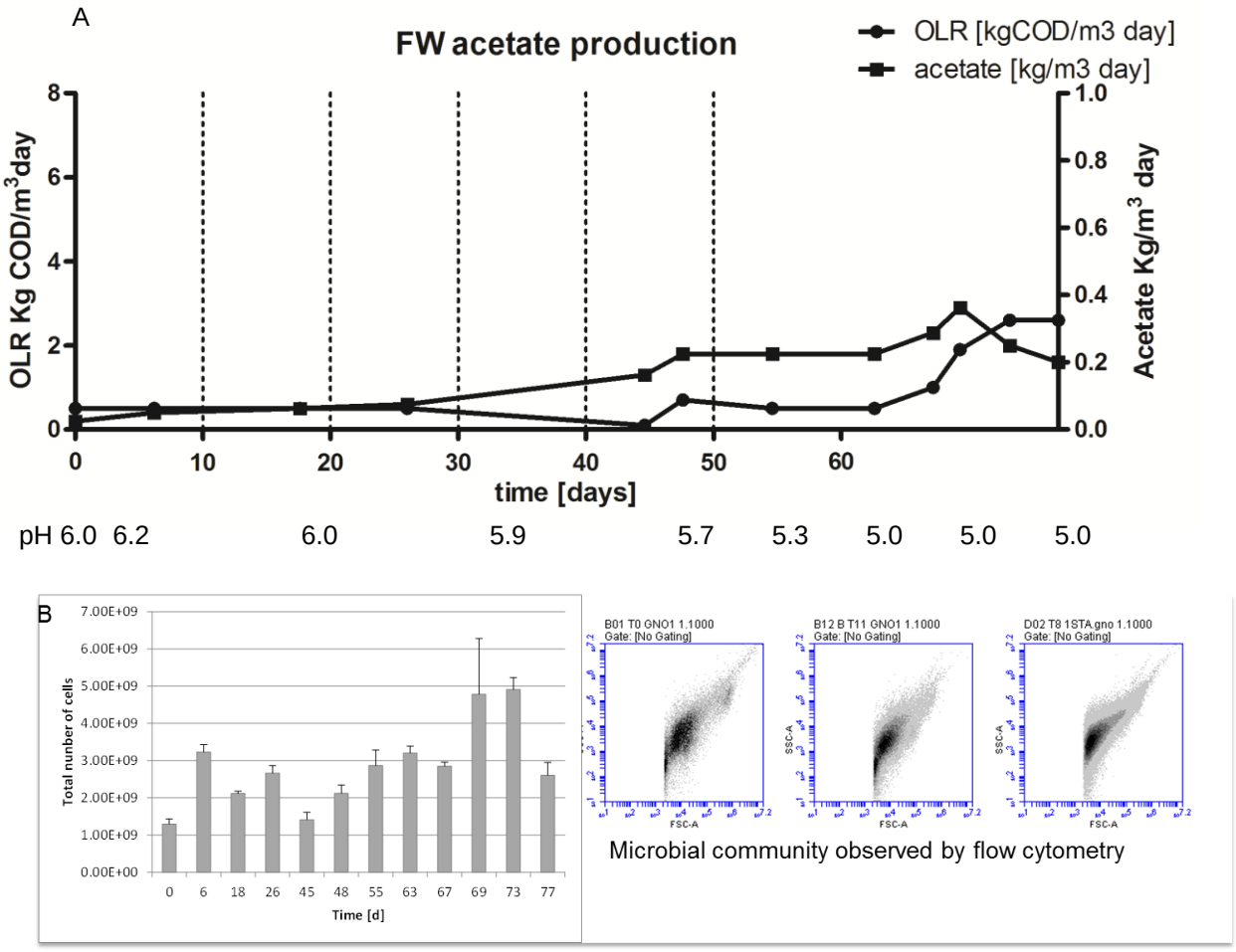


Figure 6. FWW optimal acetate production. A) concentration of acetate (black square, kg/d) and the OLR (black dots, kgCOD/ m3d) in function of the time (days). At the bottom, pH values in the sample measured every 10 days. B) Flow cytometry results, the upper figure showes the total number of cells (grey bars) along the experiment, and the bottom part showes a plot of the cellular complexity (SSC) and size (FSC) of the microbial community at the beginning, in the middle and at the end of the analysis.

The being of FWW a waste rich in oxidized sulfur residues was well evidenced the critic point of the competition among microorganisms for the same substrate, that is acetate (Annex 2). So, this experiment showed us that in laboratory conditions with small volumes (20 ml) and without any “pre-treatment” such as the use of molybdate (Annex 2), the maximal acetate production for FWW is 1.478 g of acetate every g of COD (OLR = 1.939 kgCOD/ m^3d). Anyhow, this value is expected to be increased of about 10% using 5 mM of molybdate at the beginning of the acetate production process (Annex 1): molybdate acts as antagonist of sulfate with higher affinity in the Sulfate Reducing Bacteria metabolism (see annex 2).

SK: Soapstock waste (SK) is a lipid rich solid waste from food refining industry; it is solid at room temperature and has a COD of approximately 1000 g/l and a pH around 9.5. The dilution rate at the beginning of the experiment provided a daily COD of 0.011 g/d (OLR = 0.55 kgCOD/ m^3d) until the concentration of acetate started to increase. Such an increase initiated much earlier compared to CW and FWW, just after less than 1 weeks of incubation. At this point, the daily concentration of SK was regularly increased until the production of acetate was inhibited at approximately 0.036 g/d (OLR = 1.804 kgCOD/m^3d) (Figure 7A; black dot and black square). As showed in the upper figure 7B by gray bars, the total cells number counted by FC showed a maximum value when a change in the steepness of the acetate production curve is noted (see figure 7A black arrow), and the number of cells was in the range of 10^8-10^9 cells/ml. FC showed different microbial community fingerprints compared to CW and FWW. The change of the pH in the sample from a neutral pH 6.8 to an acid pH 4.5 – 4.0 (bottom of Figure 7A) was also similar to previous results.

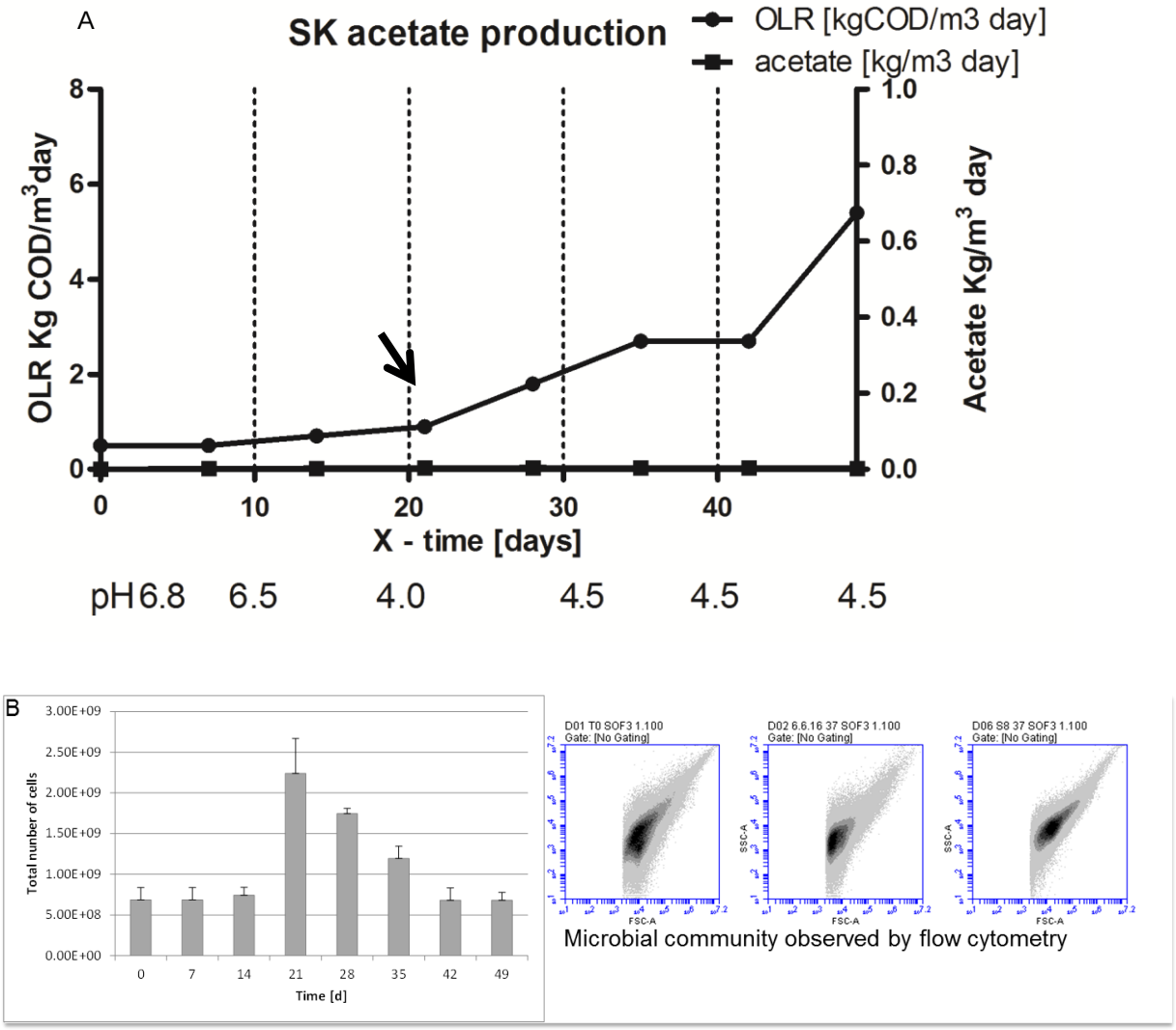


Figure 7. SK optimal acetate production. A) concentration of acetate (black square, kg/d) and the OLR (black dots, kgCOD/m^3d) in function of time (days). At the bottom of the figure the value of the pH in the sample measured every 10 days. B) Flow cytometry results, the upper graph showed the total number of cells (grey bars) along the experiment, and the bottom part showed a plot of the cellular complexity (SSC) and size (FSC) of the microbial community at the beginning, in the middle and at the end of the analysis.

SK substrate proved to be poorly solubilized in aqueous solutions, due to its high concentration of hydrophobic triglycerides. Usually, SK insolubility issue is solved by expensive biochemical pretreatment using lipases (Cherif et al. 2014). However, it should also be possible to increase SK solubility by using a strong acid such as HCl that breaks down the triglycerides, or by augmenting the temperature as for example up to 56°C. Between these two options, just the increase of temperature could raise the production of acetate (about 10x at 56°C; Annex 3), as the use of HCl results in a drastically drop of the pH in the sample (about 2.0 – 3.0, Annex 2).

This experiment showed that in laboratory conditions with small volumes (20 ml) the maximal acetate production for SK is only 0.021 g of acetate every g of COD (OLR = 1.804 kgCOD/ m^3d).

Table 7. summary results of the acetate production optimization.

Substrate	OLR kgCOD/m3 d	Max acetate production acetate/COD (g/g)
CW	5.40	0.66
FWW	1.94	1.48
SK	1.80	0.02

The comparison among the three different organic wastes showed important differences in acetate production per COD used (see table 7). In small volume bottles with 20 ml of liquid sample, best results have been obtained for FWW with an estimated production of 1.478 g of acetate per g of organic waste COD. This value is supposed to increase by about the 10% with the use of 5 mM of molybdate, which inhibits the SRB growth (Annex 2). The production of acetate by the SK substrate was really low (0.021 kgAcetate/kgCOD) compared to CW and FWW probably due to the solubility issues of the lipid rich solid waste (Annex 3).

Enrichment of the acetoclastic methanogenic community from digested sludge

Methanogens are strictly anaerobes autotrophic microorganisms, part of the archaea phylum, which possess a particularly biochemistry for methane synthesis (Thauer et al. 2008). Indeed, the coenzyme F420 (involved in methanogenesis) causes an intense auto-fluorescence of methanogenic cells under excitation by shortwave UV light. This auto-fluorescence is a diagnostic feature and can be used to check cultures of methanogens by FC and epifluorescence microscopy. The taxonomy and phylogenetic of the methanogens is based on the electron donor molecule for the methanogenesis, *e.g.* the hydrogenotrophs oxidizie H_2, the methylotrophs using methyl compound as for example methanol and the acetoclastic oxidizing acetate to produce methane (Garcia et al. 2000). In an anaerobic digester the methanogenic community was shown to count for approximately the 10% of total cells (Iannotti et al. 1978; Siebert and Hattingh 1967; Labat & Garcia 1986; O'Reilly et al. 2009).

Although the methanogens are very diverse, they can only utilize a restricted number of substrates. The substrates are limited to three major types: CO_2-methyl-group containing compounds, and acetate

(Liu, and Whitman. 2008). Within this framework, the TANAIS concept of the separation in two-phase is based on the methanogens that utilize acetate (acetoclastics).

To select acetoclastic species, part of the *Methanosarcinaceae* family, the microbial consortia has been fed only with acetate and methanol. Assuming that 1.0 g of acetate corresponds to approximately 0.78 g/l of COD, all bottles were daily loaded with 2 ml of a stock solution of 1 M acetate and 1% methanol, corresponding to a COD of 0.092 g/d (OLR = 0.306 kgCOD/m^3d). The results obtained are reported in Figure 8. The production of biogas was daily measured by the increase of the inside pressure (bar/d) as showed in the green box. The highest increase of the daily biogas production showed after about 30 days of enrichment, corresponding also to an increase of the total cell number (grey diamonds in fig. 8) and particularly of the number of auto-florescent cells (green diamonds in fig. 8) methanogens. Indeed, the acetoclastic methanogens in the sample increased from 3.4% to 6.0% at the end of the experiment, and also the methane content in the biogas shifted from approximately 50.0% from the beginning up to the 65.0% after 40 days of enrichment by acetate/methanol. At the beginning of the enrichment experiment 5 mM of molybdate has been added to prevent any SRB competition (Annex 2).

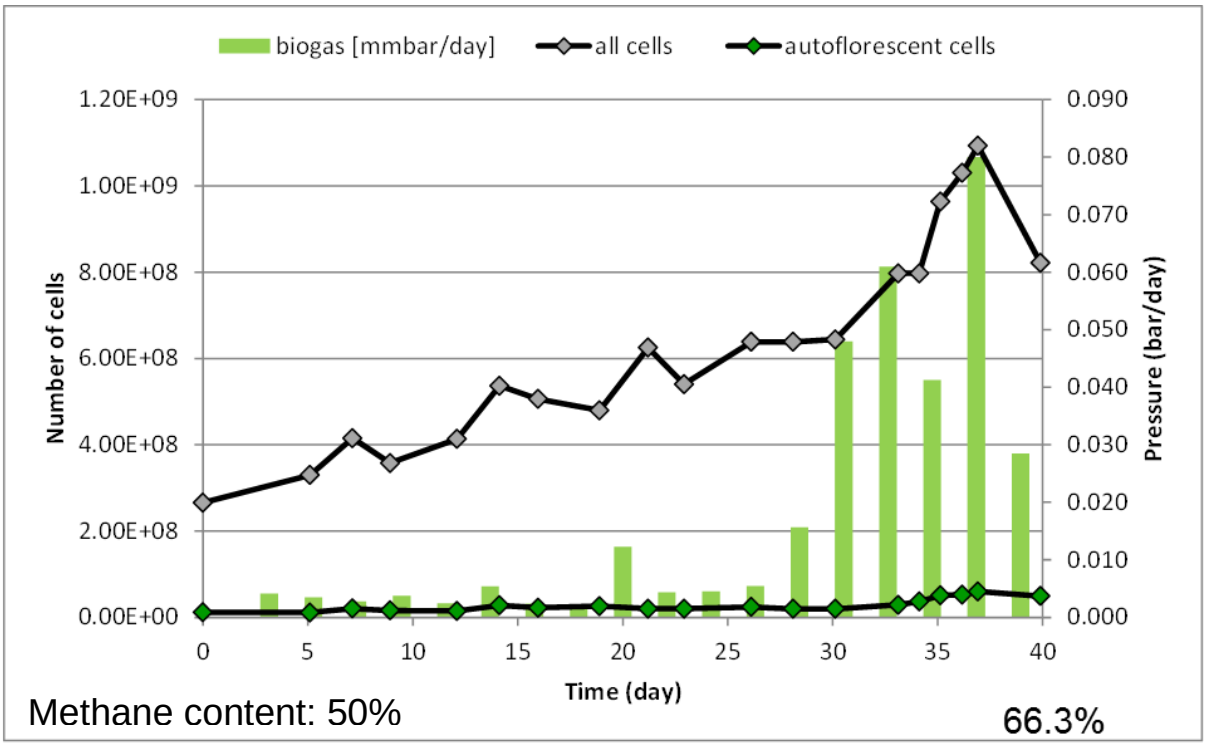


Figure 8. Enrichment of acetoclastic methanogens (Family Methanosarcinaceae).

The daily production of biogas (green bars in fig. 8), total cell number (grey diamonds in fig. 8) and auto-florescent (green diamonds in fig. 8) cell numbers have been measured for 40 days. The methane content measured at the beginning and the end of the test is reported at the bottom in fig. 8. Overall, by enrichment of acetate, the proportion of methanogens increased 3% to 6% in 40 days as measured by counting the autofluorescent cells, according to the here described enrichment procedure applied to the setting of the methanogen bioreactor (second phase bioreactor).

Increase the methanogen cells number using specific biofilm supports

The ability of growing in biofilm mode has been evaluated on the stable community of acetoclastic methanogens. Different carriers, available on the market, have been evaluated to increase the resilient active biomass in the second methanogenic bioreactor, thus avoiding continuous washout and finally increase the methane production.

Two different carriers were tested and compared to a suspended biomass sample without carriers (simply called “no carriers”). The AnoxTMK5 by Veolia (briefly named here “white carriers”) and the HX17KLL by HEL-X Biocarriers (named in the text “black carriers”) have been chosen as they are commonly in use in the wastewater treatments plants in Ticino (Figure 9, left part).

The test setting is reported in table 8. The tests have been run for 2 months of incubation. For each sample the pressure variation and methane content of the headspace has been monitored daily. Change in the microbial community composition has been evaluated by microscopic evaluation counting total cells positive to DAPI staining and methanogens by FISH with *Methanosarcinaceae* specific probe.

The daily increase of pressure inside the bottle containing the “black carries” has been about 4 time and 2 time greater at the end of the experiment (about 2 months), compared to “no carriers” and “white carriers” samples, respectively (data not shown). In the same way, also the methane content has resulted different in the 3 tests after up to 2 months of incubation with 68.0%, 73.4% and 76.3% methane increase (vol/vol) in “no carriers”, “white” and “black carriers”, respectively.

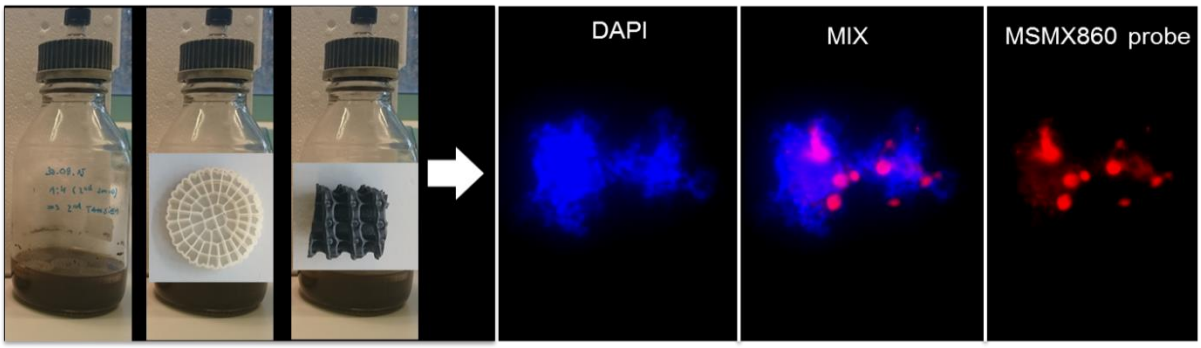


Figure 9. Setup of the methanogens distribution assessment by epifluorescent microscopy (FISH). From left to right: setup of the experiment with no carrier, white and black carrier respectively, total cells stained by DAPI, double visualization and acetoclastic methanogenic cells stained with the MSMX860 probe specific for the Methanosarcinaceae family.

The number of cells positive to DAPI and to the probe MSMX860 (specific for *Methanosarcinaceae* have been counted. Results have been reported in table 9.

Table 8. collection and sample preparation:

Biofilm carriers	Sample id	Description	Sample handling
"no carriers"	T0	Starting sample at time zero used to evaluated the growth	suspended biomass (2ml) fixed in formaldeyde and stored at -20°C
	NCs	suspended biomass in the bottle containing the "no carriers"	
"white carriers"	WCs	suspended biomass in the bottle containing the "white carriers"	
	WCa	attached biomass removed from "white carriers"	2 white carriers sonicated and PBS-washed, formaldeyde fixed and stored at -20°C
"black carriers"	BCs	suspended biomass in the bottle containing the "black carriers"	2 ml of suspended biomass fixed in formaldeyde and stored at -20°C
	BCa	attached biomass removed from "black carriers"	2 "black carriers" sonicated and PBS-washed, formaldeyde fixed and stored at -20°C

In the samples NCs "no carriers", after 2 months of incubation, the percentage of methanogen cells increased of 11.7% compared to the starting value of 5.4% (sample T0). This increased ratio was essentially due to the reduction of the total cells and not to a grow of acetoclastic methanogens. The limit of sole suspended biomass has been showed by the constant number of acetoclastic methanogens during the 2 months of experiment: indeed, once the maximum number of cells has been reached, the growth rate has been reduced towards a population numeral stability. In presence of biofilm carriers, WCs and BCs, the total cell number in the suspended biomass remained stable compared to the beginning of the experiment (T0), while the *Methanosarcinaceae* cells increased to 33% in the BCs sample and 44% in the WCs sample. The ratio between acetoclastic *Methanosarcinaceae* and total cells in the sample containing WCs has been slightly lower compared to the NCs sample, being 7.8 % and 11.7 % respectively. However, the number of acetoclastic methanogens has been recorded as higher (+42%), thus justifying the increase in methane production recorded previously (and showed above). On the other hand, in the suspended biomass samples (BCs) an increase in both the ratio and the total number of acetoclastic methanogens has been recorded: 12.2 % and 2.79 10^7 (+51%), respectively; these data further confirm the advantages of using carriers.

Moreover the total number of cells measured by DAPI has been more than 10-fold less for the attached biomass on both carriers compared to the suspended biomass. Similar results have been obtained for the number of acetoclastic methanogens, but in this case 100-fold less compared to the liquid sample. Interesting result came also on the ratio between acetoclastic *Methanosarcinaceae* and total cells, which was about two times in BCa compared to the WCa with 2.4 % and 1.3 %, respectively: this results supported the higher methane content and biogas production recorded (as showed above in the report). Actually, the percentage of acetoclastic methanogens was low compared to the liquid sample, and this was probably due to an not fully optimized protocol for cells retrieval from carriers.

The data collected clearly showed a positive effect of attached growth systems in the increased methane production, so further and more detailed experiments could deep on the knowledge of the different mechanisms involved in the increased methane production with biofilm mode methanogens.

Table 9. Epifluorescent microscopy cells counts and ratio between acetoclastic methanogens and total cells.

Sample	Total cell (DAPI) number	*Methanosarcinaceae* FISH cells number	Ratio[1]
To	$2.73x10^8$	$1.47x10^7$	5.4
NCs	$1.08x10^8$	$1.27x10^7$	11.7
WCs	$2.81x10^8$	$2.19x10^7$	7.8
WCa	$9.41x10^6$	$1.19x10^5$	1.3
BCs	$2.11x10^8$	$2.59x10^7$	12.2
BCa	$1.19x10^7$	$2.90x10^5$	2.4

[1]: percentage of *Methanosarcinaceae* in the sample

Biofilm samples have been studied also by scanning electron microscope (SEM) in order to evaluate differences in biofilm coverage of the carriers (Figure 10). Both tested carriers showed a biofilm covering the surface with no empty visible spaces. Biofilm on white carriers seemed to be entrapped in a more dense amount of EPS matrix.

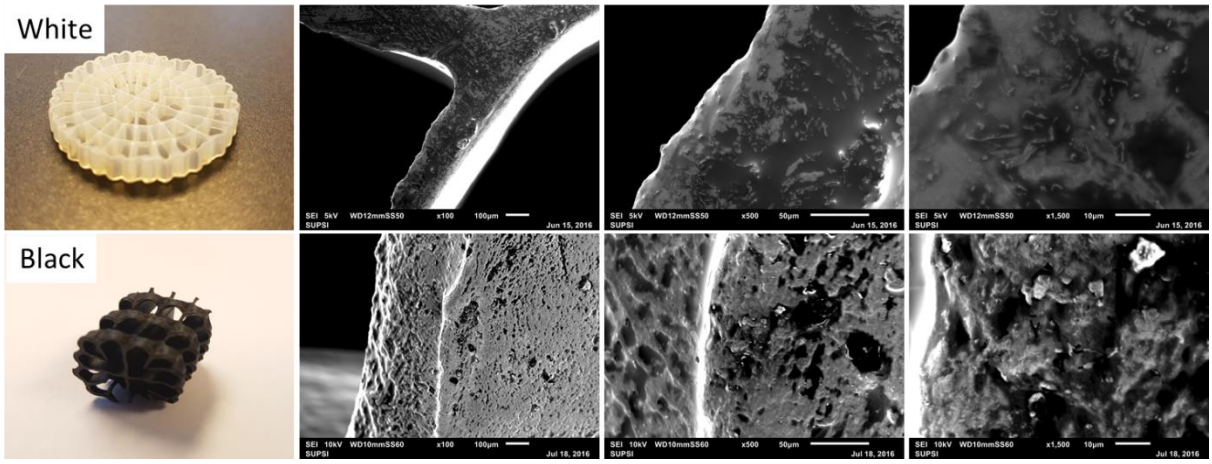


Figure 10. Evaluation of biofilm formation using 2 different carriers (SEM).

SEM images were analyzed with an image processing software to retrieve qualitative information. To have quantitative results on biofilm-surface interaction, to be able to have significant comparison, an additional technique is being evaluated. AFM Atomic Force Microscopy provides insightful data on the topography of a surface: roughness data can hence be obtained and a quantitative evaluation carried on. FM roughness analysis on biofilm slides showed however no statistical differences between biofilm samples and controls (slides with no biofilm). Further protocol redefinition will be carried for future analyses.

Startup of the methanogenesis bioreactor from the enriched laboratory cultures

The acetoclastic methanogenic microorganisms are slow growing, for this reason enrichment cultures have been set up in advance respect to the schedule. The methanogen bioreactor was first optimized by increasing the concentration of acetate/methanol until the reaching of a stable number of acetoclastic methanogens, and then used to produce methane from the acetate produced in every HAA bioreactor (CW, FWW and SK).

During the setup of the 2-stage laboratory bioreactors system, the second enriched bioreactor has been fed with a percentage (from 25 to 100 %) of acetate produced from the first organic waste analyzed such as the CW.

Some technical issues were foreseen as the methanogenic microorganisms are naturally slow-growing cells and, as risk mitigation strategy, a second “twin” bioreactor was setup in lab to be used in case of unexpected failures.

Setup the two-stages laboratory bioreactor

The setup of the two stage reactors (Figure 11) resulted more challenging than expected. In particular some technical issues caused a system failure and the restart of the process. Hereafter, the most significant arisen problems are reported together with the solving strategies adopted. Although more resources were moved to the project to avoid accumulating important delays, nonetheless only a three-weeks delay was accumulated overall. A specific design for lab scale bioreactors has been provided by the SUPSI MEMTI internal laboratory of mechanical engineering.

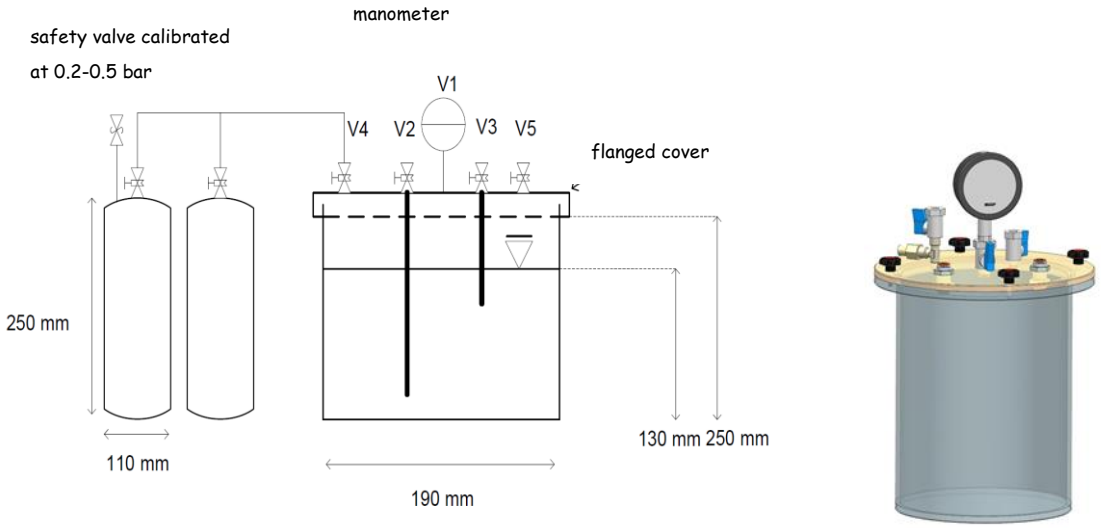


Figure 11. SUPSI lab-scale bioreactors: Piping and Instrumentation Diagram (PID) with the five hand valves for the operation with possibility of extended gas volume storage on the left and three dimension rendering of the lab scale reactor.

Reactor design technical issue: the first set of bioreactors was designed in Plexiglas, as the transparency of the material, together with the cost, was considered an important aspect to keep into consideration. Pressure tests at different temperatures showed clearly the non-suitability of the reactors as they were not pressure tight reaching the working temperature of 37°C (Figure 12).

Solving strategy adopted: A second set of bioreactors was designed and manufactured using stainless steel as material. This unexpected issue has been successfully managed with increased costs in terms of time and material.

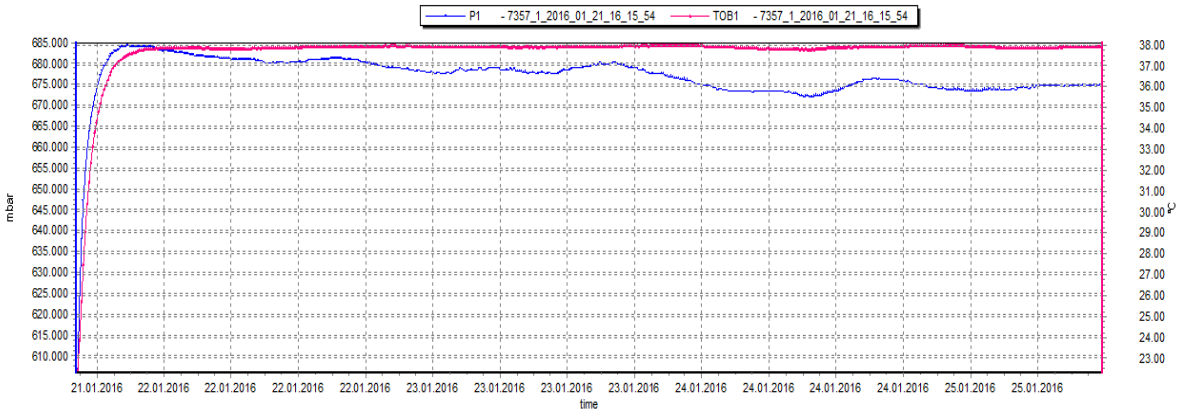


Figure 12. Sealing test of Plexiglas bioreactors. Loss of pressure (blue line) during five days testing at constant temperature values (red line).

The two-phases system was set up in its final configuration using the INOX bioreactors, using the microbial community specialized for CW in the first HAA reactor and the enrichment of the methanogen bioreactor. The system has been initially kept running manually to allow a fine control over the transition phase between the first and second steps.

"Pressure tight" technical issue: during the first week, a systematic pressure loss from reactors has been observed. **Solving strategy adopted**: a valve has been replaced and the system were left running again with manual control to verify its stability.

"Peristaltic pump compatibility" technical issue: the OLR values deemed optimal as per have been not compatible with the minimum and maximum flow rates of the peristaltic pumps. **Solving strategy adopted:** the system has been run with pure CW, run manually for the time needed to write a new programming of the automatic control (PLC) system. The system has been run for two weeks undisturbed and then methane decrease due to inhibition was observed.

"Methane inhibition" technical issue: the methane yield showed a decrease (from 50% to 30%) due to unexpected high ammonium concentration accumulated in the second reactor (NH_4 9500 mg/l). The investigated cause relayed on the feeding pace set. **Solving strategy adopted**: at first, the addition of a buffer solution as reported in (Chen et al. 2008) was tried. Because no positive results were obtained, the only chance left was restarting the system and collecting new fresh biomass directly from the digestate in Gordola plant. After three weeks of controlled feeding with 1M acetic acid solution and methanol, as soon as methane production reached 75%, the system has been fed with acetate coming from the first phase.

"Peristaltic pump" technical issue: a lack of pressure tightness caused an overflow in the second reactor due to settings of the peristaltic pumps, which were on their lower limit. **Solving strategy adopted:** The running parameters were revised, increasing the capacity of the pumps and reducing the working time. A restart of the second phase was then deemed necessary and the use of a safety

margin in the OLR to avoid the risk of system collapsing due to the complete inhibition of acetate production. The OLR was therefore set at 85% of the initially calculated value.

Summary: In the end, the system has been running stably for ten days and the process parameters were set accordingly (see figure 13): **first stage reactor: volume 0.6 liters; operative OLR 4.6 kgCOD/m^{3}d (85% of the foreseen value); reactor second stage: volume 6.5 liters of sludge and 400 introduced carrier (30% vol/vol); OLR system 0.39 kgCOD/m^{3}d**.

	I Phase			II Phase			Reactor voulme inox	
	Optimal OLR 5,4						CW COD	
OLR 0,39 gr COD/(L* Day)	Dilution	4						
	Substrate volume	0.6	L	Substrate volume	6.5	L		
	Volume inflow	0.1505112	L	Volume inflow	0.15051125	L	HRT1	3.99
	Volume inflow/day	150.5	ml	Volume inflow/giorı	150.5	ml	HRT2	43.19
	g COD/day in	2.76	gr	g COD in		gr	HRT tot	47.17
	OLR 85% of optimal	4.6		OLR System	0.39		Olr	0.39
	Carico ottimale 5,4 OLR							
OLR 0,41gr COD/(L* Day)	Dilution	3						
	Substrate volume	0.6	L	Substrate volume	6	L		
	Volume inflow	0.1316973	L	Volume inflow	0.13169734	L	HRT1	4.56
	Volume inflow/day	131.7	ml	Volume inflow/giorı	131.7	ml	HRT2	45.56
	g COD/day in	2.76	gr	g COD in		gr	HRT tot	50.11
	OLR 85% of optimal	4.6		OLR System	0.42		Olr	1.46
	Carico ottimale 5,4 OLR							
OLR 0,48gr COD/(L* Day)	Dilution	3						
	Substrate volume	0.6	L	Substrate volume	5.1	L		
	Volume inflow	0.1128834	L	Volume inflow	0.11288344	L	HRT1	5.32
	Volume inflow/day	112.9	ml	Volume inflow/giorı	112.9	ml	HRT2	45.18
	g COD/day in	2.76	gr	g COD in		gr	HRT tot	50.49
	OLR 85% of optimal	4.6		OLR System	0.48		Olr	1.45

Figure 13. operational parameters for the tests.

Defined operative protocol

- Pumping system: one cycle runs every 12 hours:
- short backwash of the system output from the second stage;
- discharge of the second phase;
- load the second stage with the substrate of the first stage;
- load of the first stage.

Evaluation of the different communities of bacteria and archaea

Due to the complexity of bacterial communities in anaerobic digestion, limited investigations have been carried out in literature to study specifics microbial profile in anaerobic digesters (Zamanzadeh et al. 2013; Zhao et al. 2016). At present, only few studies have investigated the effects on the bacterial community structure of different anaerobic digestion conditions. In this TANAIS study, the microbial profile in each bioreactor (1 single stage used as reference, 3x first HAA and 3x second methanogen) was analyzed using the Denaturing Gradient Gel Electrophoresis (DGGE). For each sample loaded, the bands pattern is an index of biodiversity and complexity of the microbial community and each band corresponds to operational taxonomic units (OTUs). This methodology has been used widely in environmental microbiology (Muyzer & Smalla 1998; Connaughton et al. 2006) to study the microbial diversity in complex systems. Here, DGGE targeting on bacterial and archaeal 16S rRNA genes, was performed to specifically monitor the microbial community composition during the two phase bioreactor methane production, for each of the three chosen organic wastes. In figure 16 the DGGE results have been reported for the three organic wastes (CW, FWW and SK) during three different metabolic moments, beginning of the feeding process with the specific organic waste (T0), the maximal methane production in the reference one stage bioreactor (TmaxOLR) and after acidification at the optimal acetate production (TmaxACE) in function of the maximal OLR for each substrate.

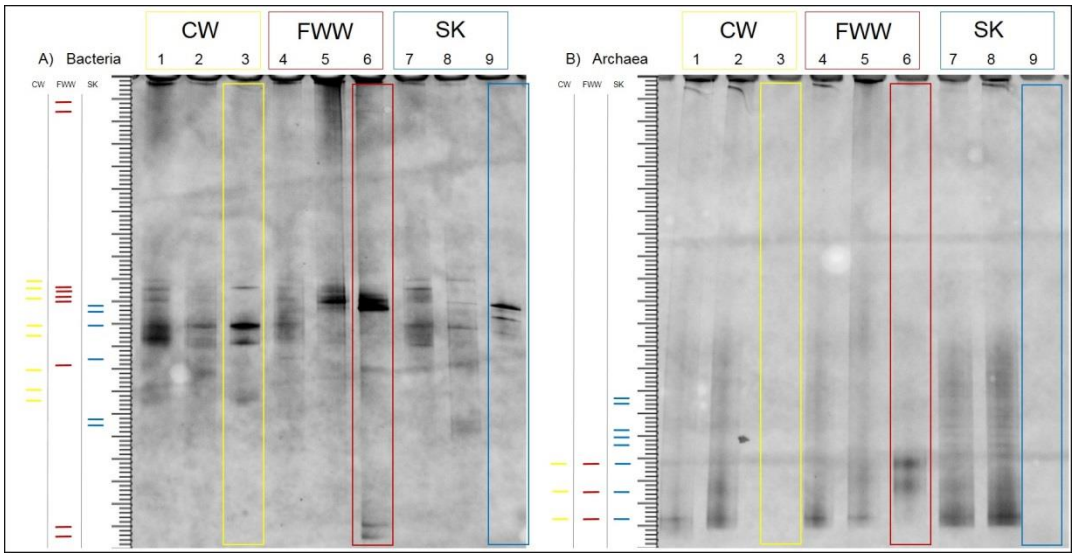


Figure 14: DGGE results for single phase reference bioreactor and optimized first HAA bioreactor. DGGE using a set of primers specific both for (A) bacteria (338f – 907rGC) and (B) archaea (ARC344fGC – ARC915r) have been used to analyze each microbial profile. Cheese whey profile, yellow marker, lines 1, 2 and 3 corresponding for T0, TmaxOLR *and Tmax ACE, respectively. Fermentation wastewater: red marker, lines 4, 5 and 6 corresponding for T0, TmaxOLR and Tmax ACE, respectively. Soapstock results: blue marker, lines 7, 8 and 9 corresponding for T0, TmaxOLR and Tmax ACE, respectively.*

In the first 2 lines for each substrate (Figure 14, 1 and 2 CW, 4 and 5 FWW and 7 and 8 for SK) the profile changed in a specific way showing an increase signal for some particular OTUs. These changes, particularly in the bacterial communities' profile (figure 15), corresponded to the specialization of the microorganisms for each substrate, indeed every TmaxOLR had a precise profile

defined by the specific substrate composition. The diversity in the archaeal communities (Figure 16) has been not so remarkable as showed before for bacteria, indeed similar profiles have been showed for each particular substrate reflecting an enrichment of acetoclastic methanogens in the bioreactor, confirming the enrichment process previously reported.

The microbial profiles then completely changed for the third line (Tmax ACE) for each organic waste: indeed, the optimization of the acetate production kept only few OTUs stable and similar to others two lines. This change is probably due to the drastic environmental changes such as the acidification of the pH from 7.0 to 4.0, which selected for other better adapted microorganisms (Figure 15). Interesting also the disappearance of all methanogens for the CW (figure 16, line 3) and the SK (Figure 16, line 9) outselected probably by the acid pH. The microbial profile of the FWW at the maximal production of acetate (Figure 16, line 6) showed the presence of some archaeal OTUs that can be explained with the pH condition (5.0) being not acid enough to significantly eliminate all methanogens in the sample.

During the quantification of methane production, in the laboratory two phase bioreactor, the microbial communities stability was evaluated at the beginning and at the end of the measurement for each organic waste. Moreover, the profiles in the first HAA bioreactor showed similarities to those shown in figure 14 (line 3, 6 and 9) for the optimal production of acetate (TmaxACE).

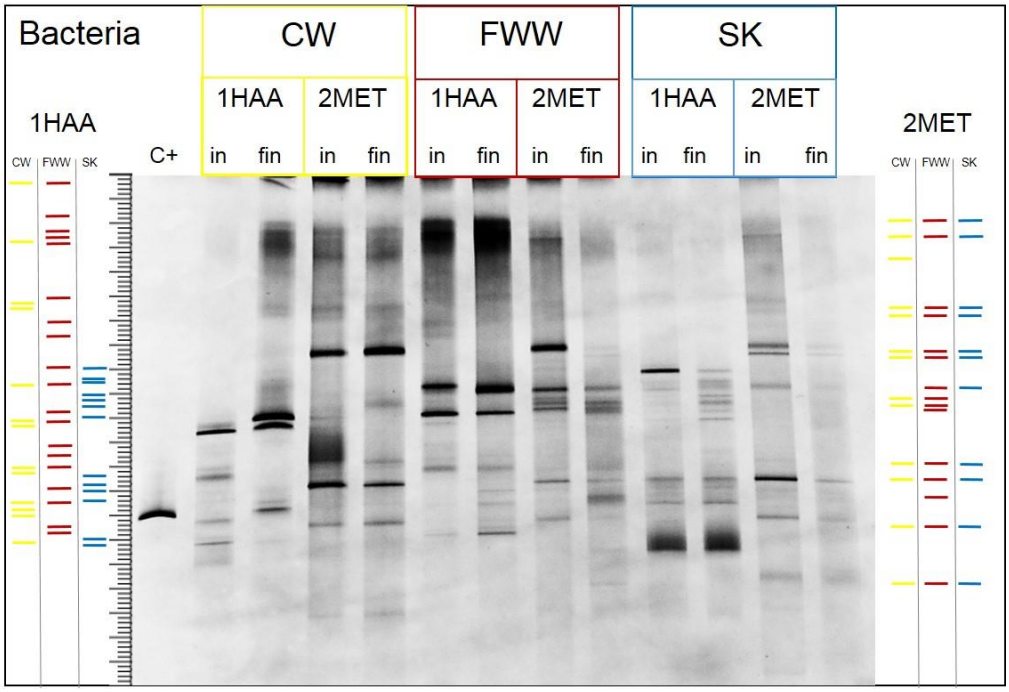


Figure 15: bacteria DGGE results from the two phases bioreactor. DGGE using a set of primers specific for bacteria (338f – 907rGC) has been used to analyze the microbial communities at the beginning (in) and at the end (fin) during the methane quantification). Microbiological community structure has been showed in the first HAA and in the second methanogens (MET) bioreactors. Each organic waste were characterized by a specific color: yellow, red and blue for CW, FWW and SK respectively. Positive control (C+) correspond to a pure culture of Escherichia coli.

The bacterial profiles at the beginning and at the end for each organic waste has been reported in figure 15. As expected, completely different profiles have been reported for each first phase HAA bioreactor (1HAA) for CW, FWW and SK: specific microbial communities adapted to the main

characteristic of each given substrate. Initial (in) and final (fin) bacterial profiles for FWW and SK did remain almost the same along the period of methane production confirming the stability of the process of acetate production. However, for CW the initial (in) bacterial profile showed some difference compared to the final (fin), this being probably due to the steep increase of the ammonium (NH_4^+) concentration up to 7 g/l resulting in unexpected stress factor to the microbial community during the quantification of the methane production.

The comparison among microbial profiles for the maximal acetate (Tmax ACE) production (Figure 15) resulted difficult for the first HAA bioreactor during the quantification of methane production (Figure 16). However, the similarity of the microbial profile at the beginning and at the end suggested a stability in the acetate production process during methane quantification experiment.

On the other hand, similar bacterial profiles have been observed for the second methanogen bioreactor (2MET) confirming the selection of a stable methanogenic community on the organics from the first phase (*e.g.* VFA). The proved stability of the microbial biomass in the second phase reactor is important for a constant production of methane.

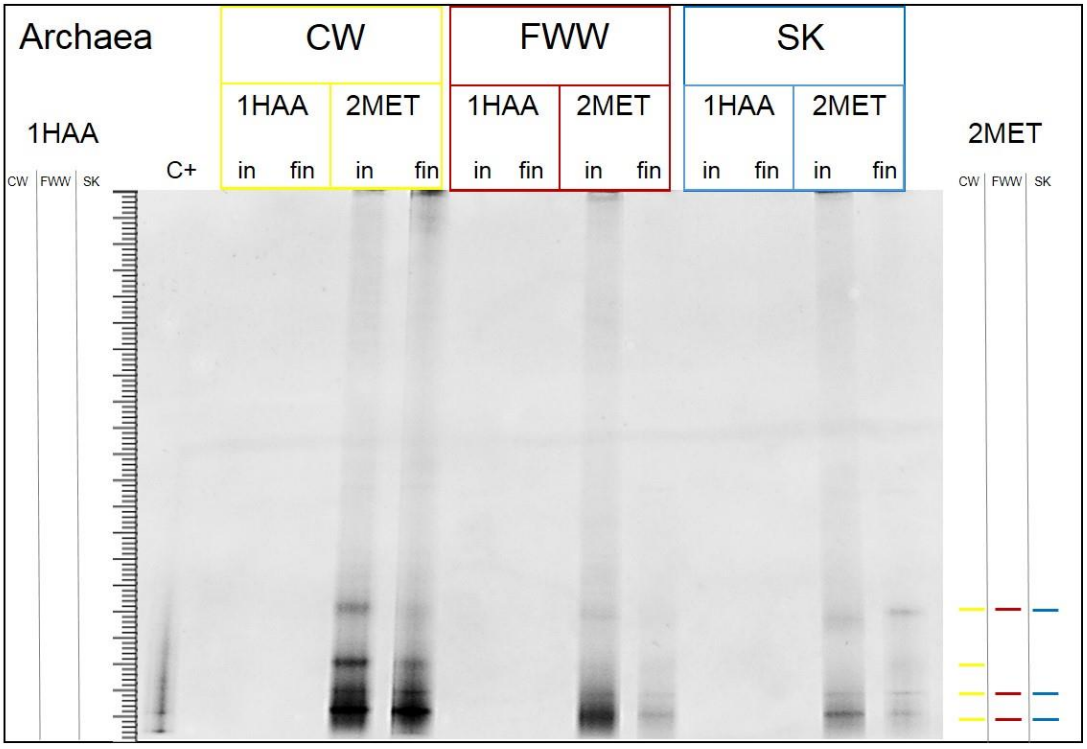


Figure 16: archaeal DGGE results from the two phases bioreactor. DGGE using a set of primers specific for archaea (ARC344fGC – ARC915r) has been used to analyze the microbial communities at the beginning (in) and at the end (fin) during the methane quantification (task 1.6). Microbiological community structure has been shown in the first HAA and in the second methanogens (MET) bioreactors. Each organic waste has been characterized by a specific color: yellow, red and blue for CW, FWW and SK respectively. Positive control (C+) correspond to a pure culture of acetoclastic methanogen Methanosarcina barkeri.

In figure 16 methanogens band profile has been reported evidencing the stability of the second methanogen bioreactor (2MET) during the entire process of methane production for each organic

waste (from beginning November to end March). Similar results have been observed for bacteria (Figure 15), all the archaeal profiles showed almost the same OTUs for every specific substrate. This result confirmed a stable structured microbial community for the methanogens in a two phases setup, compared to a typical one phase. Indeed, the archaeal profile for the reference one phase bioreactor (Figure 16) showed differences in the band profile.

In the first HAA bioreactor, *i.e.* the specialized on the production of acetate, the archaea have been not detected, as they were not detected for CW and SK (Figure 14). The lack of methanogens also for the FWW substrate has been probably due to prolonged incubation at low pH of 5.0, which strongly inhibits on longer time archaeal cells.

Monitoring microbial profiles in anaerobic digestion bioreactors can offer important hints on slow changes happening in the composition of the microbial community that is affected by even subtle changes in the environment (*i.e.* reactor). This powerful tool can help managing reactors together with standard monitoring procedures to improve reliability, particularly for use in large-scale installations.

Quantification of the biomethane production in the two-phase process

The methane production for each substrate (CW, FWW and SK) have been evaluated during a period of about 30 days and then compared with values recorded in the one-stage bioreactor.

To compare, in a robust way, data from single reactor with the TANAIS process data, the operational parameters (OLR and dilution rate) giving best yields in single stage have been chosen for each substrate. Nevertheless, as whey has shown really promising results, different OLRs have been tested in addition with the aim to pin point differences in the process performance.

The methane yield tests have been designed with no replicates, but with a start-up phase carefully monitored to guarantee the compliance with the chosen operational parameters.

Cheese Whey CW. In the start-up phase 0.39 kgCOD/m^{3}d OLR of the whole system has been obtained diluting the whey with water (whey:water = 1:4); this condition led to a 4.6 kgCOD/m^{3}d OLR for the HAA reactor (first phase) value that corresponded to the 85% of the optimal OLR to produce acetic acid. HRT have been set to 3.99 and 47.1 days for the first and second phase, respectively.

The second OLR tested has been 0.48 kgCOD/m^{3}d obtained by diluting the mixture whey:water = 1:3 and keeping constant the OLR for the first phase to the set value of 4.6 kgCOD/m^{3}d and HRTs equal to 5.3 for the first phase and a total HRT of 50.4 days.

Table 10. Methane production of CW in the two phase system.

	Methane production		
OLR $\frac{Kg\ COD}{m^3\ d}$	$\frac{Nm^3}{m^3 CW}$	$\frac{Nm^3}{Ton\ COD}$	$\frac{Nm^3}{Ton\ VS}$
0,39	**27,1**	**369,3**	**932,4***
0,48	**17**	**232,4**	**586,8***

***these values are affected by heterogeneity of the CW as fats are not evenly distributed.**

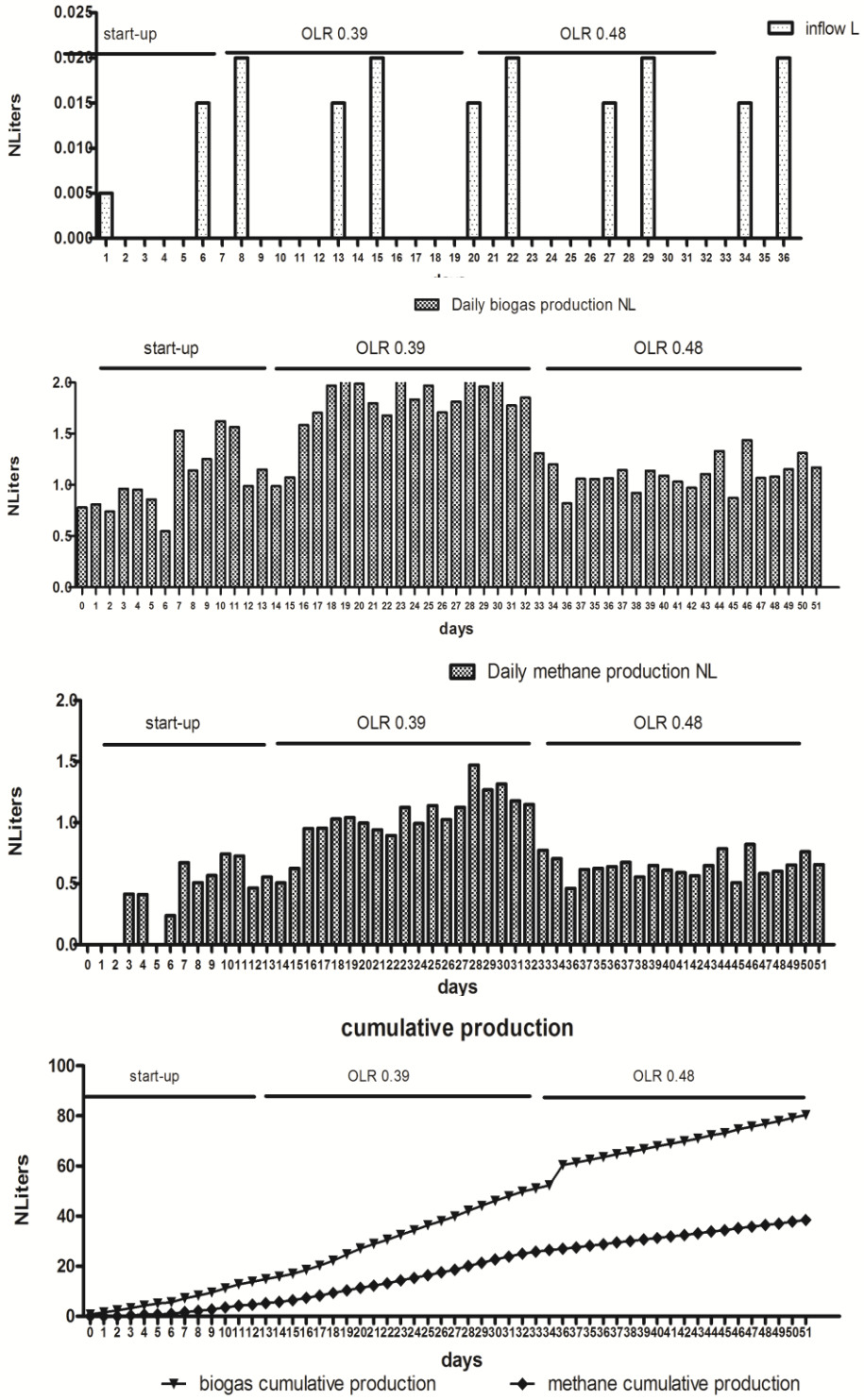


Figure 17. Performance of two-stage bioreactor. From top to bottom: feeding of CW, biogas and methane daily and cumulative production in the bioreactor.

The first bioreactor produced acetic acid in a constant and continuous mode allowing for a global stable process with abundant methane production.

The two phases reached stability in short time with pH values around 3 for the HAA and 7.5 for the methanogenic reactor, respectively. Key parameters of whey digestion are reported in table 11.

Table 11. Key parameters of two-stage bioreactor fed by CW.

OLR $\frac{Kg\ COD}{m^3\ d}$	First phase				Second phase		
	pH	**Acetic Acid (mg/l)**	**COD (mg/l)**	**Alkalinity (mg/l)**	**pH**	**Acetic Acid (mg/l)**	**COD (mg/l)**
0,39	3,53	2700	14800	2870	7,53	2300	5000
	3,38	6830*	24090*	3040*	7,17	1050*	9240*
	3,37	3130	18030	2450	7,52	1170	3030
	3,37	3320	18700	2550	7,52	504	1500
0,48	3,31	3790	17100	2691	8,05	571	4408
	3,3	4060	2200	2490	7,56	547	4200

*these values are affected by sample filtration issue.

Biogas produced had a content of minimum 50% with a peak value of 66%, CO_2 37-40% and H_2S concentration less than 270 ppm with mean values around 200 ppm.

Fermentation WasteWaters FWW. The start-up phase has been carried out separately for the two reactors (HAA and methanogenic) feeding them with FWW and acetic acid, respectively. Subsequently, the two reactors were connected and process stability has been verified for a ten days-time frame. The operational parameters set for the tests have been those giving best results in single phase process, namely 0.40 kgCOD/m^3d OLR, with FWW 1:10 diluted in water as feeding. The OLR in the HAA reactor has been kept at 1.7 kgCOD/m^3d, allowing for a HRT of 9.4 days in the first phase and 39.5 days as HRT for the whole process. The results obtained for FWW are reported in table 12.

Table 12. Methane production of FWW in the two phases system.

	Methane production		
OLR $\frac{Kg\ COD}{m^3\ d}$	$\frac{Nm^3}{m^3 FWW}$	$\frac{Nm^3}{Ton\ COD}$	$\frac{Nm^3}{Ton\ VS}$
0,40	**16,9**	**103,9**	**58,7**

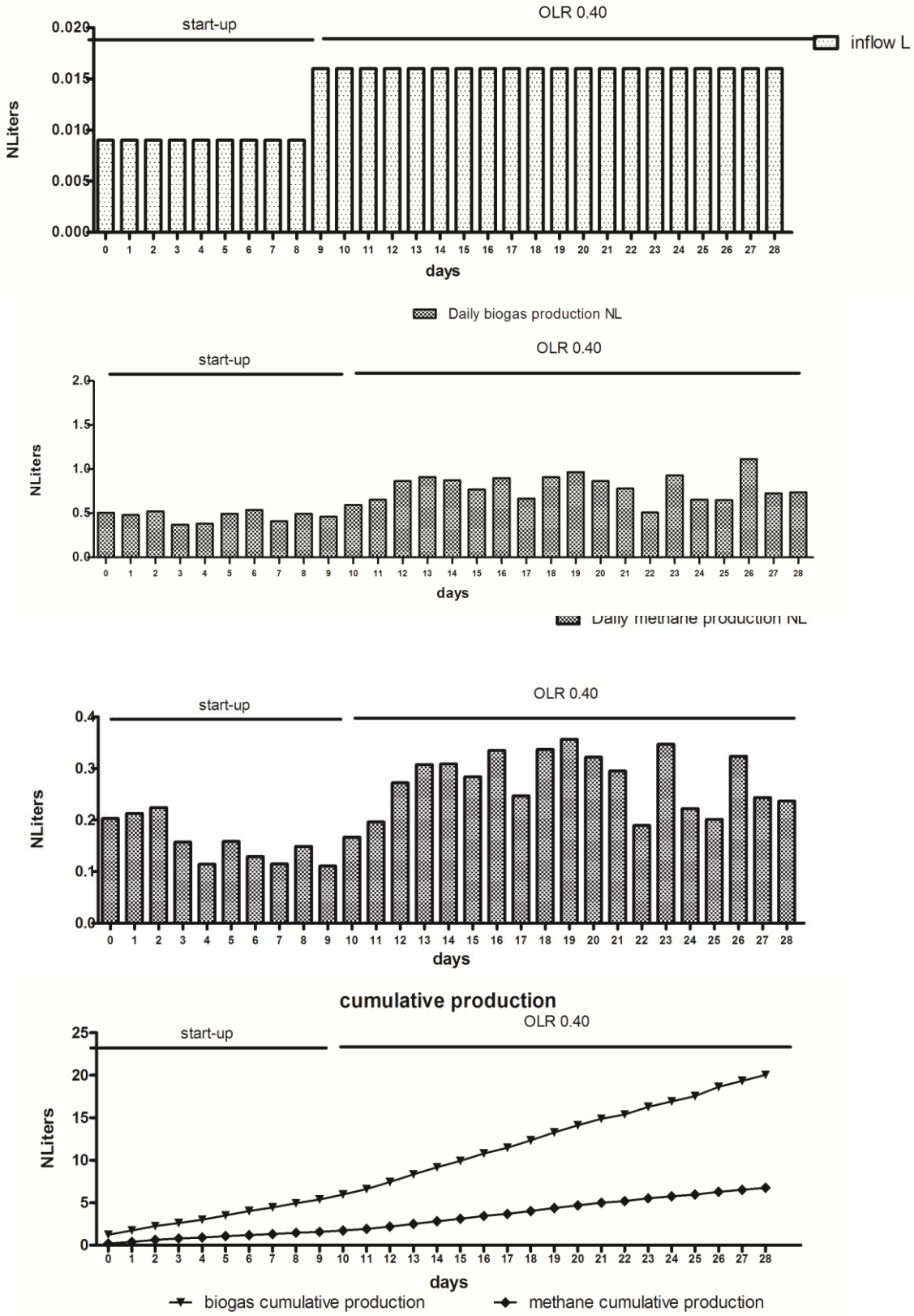


Figure 18. Performance of two-stage bioreactor. From top to bottom: feeding of FWW, biogas and methane daily and cumulative production in the bioreactor.

The results obtained for the two phases process showed a stable production of biogas and methane; comparing the data with the single phase reference a decrease in the volume of produced methane has been measured. The first HAA reactor produced large amounts of acetic acids and these data were promising: however, a lack of corresponding methane was assessed. Methane percentages were below 37%, while H_2S were higher than the upper detection limit of the instrument (600 ppm). Most likely the high H_2S concentration caused an inhibition in the methanogenic metabolism.

Stable processes have been rapidly reached with pH values of 4.46 e 7.27 for the first and second phase, respectively. The high COD values in the output (11000-21000 ppm) is due to a partial failure of the methanogenic reactor to completely convert produced acetate. Alkalinity measured as $CaCO_3$ is buffering the system during the whole process avoiding unwanted acidification.

Table 13. Key parameters of two-stage bioreactor fed by FWW.

OLR $\frac{Kg\ COD}{m^3\ d}$	First phase				Second phase		
	pH	**Acetic Acid** (mg/l)	**COD** $(\mathbf{mg/l})$	**Alkalinity** (mg/l)	**pH**	**Acetic Acid** (mg/l)	**COD** $(\mathbf{mg/l})$
0,40	4,35	10890	>22000	6010	7,23	5000	11000
	4,34	10600	>22000	6390	7,44	6390	17000
	4,58	11760	>22000	4530	7,30	8450	21000

The partial failure to convert acetic acids to methane by methanogens observed in the second reactor is due to the simultaneous presence of oxidized sulphur compounds and organics, that can be utilized by Sulphur Reducing Bacteria (SRB). The presence of sulphates shift the balance in favor of the competitor SRB against methanogens. Moreover, the metabolic product of SRB respiration (namely H_2S) is a toxic compound which, in high concentrations, inhibits methanogens and others microorganisms.

Soapstocks SK: The start-up phase has been carried out separately for the two reactors (HAA and methanogenic) feeding them with SK and acetic acid, respectively. Subsequently, the two reactors were connected and the process stability has been verified for a thirteen days-time frame. The operational parameters set for the tests have been the ones that gave best results in single phase process, namely OLR 0.27 kgCOD/m^3d. and SK diluted in water in a 1:100 ratio.

The HAA reactor has been set with the operational parameters previously defined (see “Monitoring and optimizing of acetate production rates and operation parameters”) that were: ORL 1.53 kgCOD/m^3d and HRT equal to 7.1 days. The HRT of the whole system has been set to 39.6 days. The results obtained for SK are reported in table 14 and in the figure 19.

Table 14. Methane production of SK in the two phases system.

	Methane production		
OLR $\frac{Kg\ COD}{m^3\ d}$	$\frac{Nm^3}{m^3 CW}$	$\frac{Nm^3}{Ton\ COD}$	$\frac{Nm^3}{Ton\ VS}$
0,27	**26,9**	**24,9**	**79,5**

The solubility issue, that was evidenced during the single-phase tests, came across also in the two phase tests. The biogas yield has been low even though the methane content in the biogas was greater than 50% (mean value 54.3% and max value 57.5%). The acetic acid production in the first phase is low compared to the potential value due to the organic load of the substrate; mean acetic acid concentration is lower than 500 ppm and the pH did not reach the characteristic acid values (min pH 6.06). The pH of the second phase was stable around 8.0 due to the unbalanced buffer capacity of alkalinity.

Table 15. Key parameters of two-stage bioreactor fed by SK.

OLR $\frac{Kg\ COD}{m^3\ d}$	**First phase**				**Second phase**		
	pH	**Acetic Acid** (mg/l)	**COD** (mg/l)	**Alkalinity** (mg/l)	**pH**	**Acetic Acid** (mg/l)	**COD** (mg/l)
0,27	6,18	471	1700	2910	8,25	2400	3770
	6,24	391	2160	6830	8,22	1830	2630
	6,06	249	1660	5140	7,92	1520	2770
	6,63	339	1720	4220	8,07	1210	2380

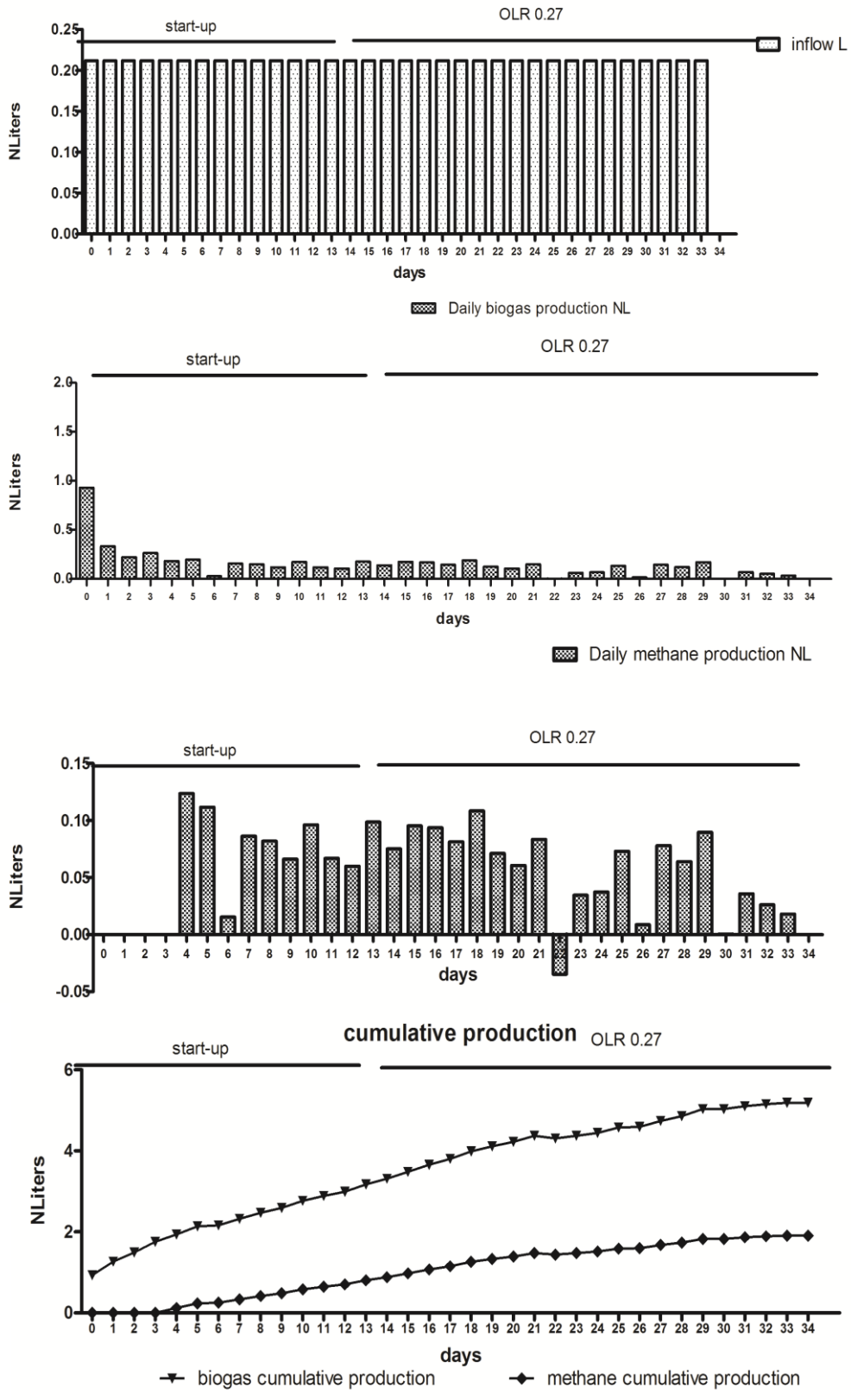


Figure 19. Performance of two-stage bioreactor fed by SK. From top to bottom: feeding of SK, biogas and methane daily and cumulative production in the bioreactor.

Conclusions and Outlook

The three different industrial wastes, all of interest for industries from Ticino, have been tested for methane production applying a two phases process that allows a better managing of the microbial community responsible for the process itself.

These three substrates have very different characteristics in terms of organic content, presence of potential inhibitor and solubility: they indeed responded to the two phases process in different ways. **The first substrate tested -cheese whey- gave the most promising results, almost doubling the methane yield with respect to the single stage process, with up to 369 Nm^3/TCOD versus 175 Nm^3/TCOD as reported in table 15**.

The second substrate tested -fermentation wastewater- has good COD content and common chemical characteristics, but the high concentration of sulphur compounds, coupled with the organic content, unbalanced the microbial equilibrium thus favoring the SRBs, which are natural competitors of methanogens and produced the toxic hydrogen sulfide.

The third substrate tested has been soapstocks. This waste has been known as a problematic substrate due to the solubility issue that has not been solved even by a specific separated first phase that combines hydrolysis, acidogenesis and acetogenesis in the two stage bioreactor.

Table 15. comparative results obtained for the TANAIS process for the three substrates tested.

		Single stage reference		**Two phase TANAIS**	
				Methane yield	
	OLR	Nm^3/m^3substrate	Nm^3/T COD	Nm^3/m^3substrate	Nm^3/T COD
Cheese whey CW	0.39	**10.9±0.9**	175± 15	**27.1**	369.3
Fermentation Wastewaters FWW	0.40	43,9 ± 6.2	274±39	16.6	103.9
Soapstocks SK	0.27	88,9 ± 16,4	82,1 ± 15,2	26.9	24.9

The main TANAIS Milestone was the achievement of an increase of at least 30% in methane production for at least one substrate, compared to a traditional single phase process.

The data collected for CW fulfill this requirement. The data are however obtained on lab-scale system, the tests were carried out in without replicates and the feeding done from a stock sample was not completely representative of the fluctuations in the chemical characteristics of an industrial byproduct. Due to these limits further tests done in a scale up system would be highly recommended to further improve the robustness of the data and hence strengthen these promising results. A scale up of the process specifically for the whey is indeed being planned in order to validate the results obtained so far.

The focus of the next investigation will be on the scale up of the system, but different options could be investigated to overcome the limits of the FWW and SK. The questions still open are the evaluation of specific inhibitor such as the molybdate to reduce the hydrogen sulfide production provided by the SRBs, the option to strengthen the hydrolysis step using for example specific enzymes or fungi for the solubility issue and the investigation on carriers role on biofilm-mode methanogenesis. For sake of completeness, these open questions are briefly discussed in the annexes.

References

APHA. Standard methods for the examination of water and wastewater, 21sted. Washington, DC, New York: American Public Health Association; 2005.

Barton LL & Fauque GD (2009) Chapter 2 Biochemistry, Physiology and Biotechnology of Sulfate-Reducing Bacteria. Adv. Appl. Microbiol. 68: 41–98.

Bertin L, Grilli S, Spagni A & Fava F (2013) Innovative two-stage anaerobic process for effective codigestion of cheese whey and cattle manure. Bioresour. Technol. 128: 779–783. http://www.ncbi.nlm.nih.gov/pubmed/23186682 (Accessed June 9, 2014).

Biswas KC, Woodards NA, Xu H & Barton LL (2009) Reduction of molybdate by sulfate-reducing bacteria. BioMetals 22: 131–139. http://link.springer.com/10.1007/s10534-008-9198-8 (Accessed June 30, 2016).

Bottinelli M (2008) Approche moléculaire à l'étude des bactéries sulfato-réductrices et des Archaea méthanogènes dans les sédiments des lacs Cadagno et Rotsee. Section des Sciences de la Terre, Université de Genève, Genève.

Chen Y, Cheng JJ & Creamer KS (2008) Inhibition of anaerobic digestion process: A review. Bioresour. Technol. 99: 4044–4064.

Cherif S, Aloui F, Carrière F & Sayadi S (2014) Lipase Pre-Hydrolysis Enhance Anaerobic Biodigestion of Soap Stock from an Oil Refining Industry. J. Oleo Sci. J. Oleo Sci 63: 109–114.

Connaughton S, Collins G & O'Flaherty V (2006) Development of microbial community structure and actvity in a high-rate anaerobic bioreactor at 18°C. Water Res. 40: 1009–1017.

Cooney M, Maynard N, Cannizzaro C & Benemann J (2007) Two-phase anaerobic digestion for production of hydrogen-methane mixtures. Bioresour. Technol. 98: 2641–2651. http://www.ncbi.nlm.nih.gov/pubmed/17169554 (Accessed February 18, 2015).

Enning D & Garrelfs J (2014) Corrosion of Iron by Sulfate-Reducing Bacteria: New Views of an Old Problem. Appl. Environ. Microbiol. 80: 1226–1236. http://aem.asm.org/cgi/doi/10.1128/AEM.02848-13 (Accessed June 28, 2016).

Muyzer EC de W and AGU (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes.

Garcia J-L, Patel BK. & Ollivier B (2000) Taxonomic, Phylogenetic, and Ecological Diversity of Methanogenic Archaea. Anaerobe 6: 205–226. http://linkinghub.elsevier.com/retrieve/pii/S1075996400903457 (Accessed July 1, 2016).

Glöckner FO, Amann R, Alfreider A, Pernthaler J, Psenner R, Trebesius K & Schleifer K-H (1996) An In Situ Hybridization Protocol for Detection and Identification of Planktonic Bacteria. Syst. Appl. Microbiol. 19: 403–406. http://linkinghub.elsevier.com/retrieve/pii/S0723202096800695 (Accessed February 11, 2014).

Iannotti EL, Fischer JR & Sievers DM (1978) Medium for the enumeration and isolation of bacteria from a Swine waste digester. Appl. Environ. Microbiol. 36: 555–566. http://www.ncbi.nlm.nih.gov/pubmed/16345317 (Accessed July 1, 2016).

de Jesus EB, de Andrade Lima LRP, Bernardez LA & Almeida PF (2015) INHIBITION OF MICROBIAL SULFATE REDUCTION BY MOLYBDATE. Brazilian J. Pet. Gas 9: 95–106. http://www.portalabpg.org.br/bjpg/index.php/bjpg/article/view/435 (Accessed June 28, 2016).

Kraemer JT & Bagley DM (2007) Improving the yield from fermentative hydrogen production. Biotechnol. Lett. 29: 685–695. http://www.ncbi.nlm.nih.gov/pubmed/17279447 (Accessed February 23, 2015).

Labat M & Garcia JL (1986) Study on the development of methanogenic microflora during anaerobic digestion of sugar beet pulp. Appl. Microbiol. Biotechnol. 25: 163–168. http://link.springer.com/10.1007/BF00938941 (Accessed July 1, 2016).

Liu, Yuchen, and William B. Whitman. 2008. “Metabolic, Phylogenetic, and Ecological Diversity of the Methanogenic Archaea.” Annals of the New York Academy of Sciences 1125: 171–89. doi:10.1196/annals.1419.019.

Liu D, Liu D, Zeng RJ & Angelidaki I (2006) Hydrogen and methane production from household solid waste in the two-stage fermentation process. Water Res. 40: 2230–2236. http://www.sciencedirect.com/science/article/pii/S0043135406002004 (Accessed November 27, 2014).

Moter, Annette, and Ulf B. Göbel. "Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms." (2000): 85-112.

Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73: 127–141. http://www.ncbi.nlm.nih.gov/pubmed/9602286.

O’Reilly J, Lee C, Collins G, Chinalia F, Mahony T & O’Flaherty V (2009) Quantitative and qualitative analysis of methanogenic communities in mesophilically and psychrophilically cultivated anaerobic granular biofilims. Water Res. 43: 3365–3374.

Office of Water O of (2006) Biosolids technology fact sheet: multi-stage anaerobic digestion. EPA United State Environ. Prot. Agency.

Rabus R, Hansen TA & Widdel F (2013) Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes. The Prokaryotes, pp. 309–404. Springer Berlin Heidelberg, Berlin, Heidelberg http://link.springer.com/10.1007/978-3-642-30141-4_70 (Accessed June 30, 2016).

Raskin L, Stromley JM, Rittmann BE & Stahl DA (1994) Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol. 60: 1232–1240. http://www.ncbi.nlm.nih.gov/pubmed/7517128 (Accessed June 21, 2016).

Reis MAM, Lemos PC, Almeida JS & Carrondo MJT (1990) Influence of produced acetic acid on growth of sulfate reducing bacteria. Biotechnol. Lett. 12: 145–148. http://link.springer.com/10.1007/BF01022432 (Accessed June 28, 2016).

Saddoud A, Hassaïri I & Sayadi S (2007) Anaerobic membrane reactor with phase separation for the treatment of cheese whey. Bioresour. Technol. 98: 2102–2108. http://www.sciencedirect.com/science/article/pii/S0960852406004238 (Accessed February 3, 2015).

Shah FA & Mahmood Q (2014) Microbial ecology of anaerobic digesters: the key players of anaerobiosis. Sci. World …. http://www.hindawi.com/journals/tswj/2014/183752/abs/ (Accessed February 5, 2015).

Siebert M & Hattingh W (1967) Estimation of methane-producing bacterial numbers by the most probable number (MPN) technique. Water Res. 1: 13–19. http://linkinghub.elsevier.com/retrieve/pii/0043135467900607 (Accessed July 1, 2016).

Sieger R (2001) Anaerobic digestion: Revitalized. The Straight Scoop on Biosolids 3–6. https://scholar.google.ch/scholar?hl=it&q=+The+Straight+Scoop+on+Biosolids&btnG=&lr=#0 (Accessed February 5, 2015).

Stams AJM, Plugge CM, Bok FAM de, Houten BHGW van, Lens P, Dijkman H & Weijma J (2005) Metabolic interactions in methanogenic and sulfate-reducing bioreactors. Water Sci. Technol. 52: 13–20.

Storelli N (2014) Role of phototrophic sulfur bacteria from the chemocline in the primary production of Lake Cadagno. http://archive-ouverte.unige.ch/unige:34915 (Accessed May 19, 2015).

Takahashi, Chisato, et al. "Electron microscopy of Staphylococcus epidermidis fibril and biofilm formation using image-enhancing ionic liquid." *Analytical and bioanalytical chemistry* 407.6 (2015): 1607-1613.

Thauer RK, Kaster A-K, Seedorf H, Buckel W & Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6: 579–591. http://www.ncbi.nlm.nih.gov/pubmed/18587410 (Accessed May 23, 2014).

Ueno Y, Fukui H & Goto M (2007) Operation of a two-stage fermentation process producing hydrogen and methane from organic waste. Environ. Sci. Technol. 41: 1413–1419. http://pubs.acs.org/doi/abs/10.1021/es062127f (Accessed February 18, 2015).

Whitman, William B., Timothy L. Bowen, and David R Boone. 2006. “The Methanogenic Bacteria.” In The Prokaryotes: Volume 3: Archaea. Bacteria: Firmicutes, Actinomycetes, edited by Martin Dworkin, Stanley Falkow, Eugene Rosenberg, Karl-Heinz Schleifer, and Erko Stackebrandt, 165–207. New York, NY: Springer New York. doi:10.1007/0-387-30743-5_9.

Zamanzadeh M, Parker WJ, Verastegui Y & Neufeld JD (2013) Biokinetics and bacterial communities of propionate oxidizing bacteria in phased anaerobic sludge digestion systems. Water Res. 47: 1558–1569. http://www.sciencedirect.com/science/article/pii/S0043135412008998 (Accessed April 5, 2017).

Zhao Z, Zhang Y, Ma W, Sun J, Sun S & Quan X (2016) Enriching functional microbes with electrode to accelerate the decomposition of complex substrates during anaerobic digestion of municipal sludge. Biochem. Eng. J. 111: 1–9. http://www.sciencedirect.com.proxy2.biblio.supsi.ch/science/article/pii/S1369703X16300663 (Accessed April 5, 2017).

Zhu H, Parker W, Conidi D, Basnar R & Seto P (2011) Eliminating methanogenic activity in hydrogen reactor to improve biogas production in a two-stage anaerobic digestion process co-digesting municipal food waste and sewage sludge. Bioresour. Technol. 102: 7086–7092. http://www.sciencedirect.com/science/article/pii/S096085241100561X (Accessed February 18, 2015).

Zhu H, Stadnyk A, Béland M & Seto P (2008) Co-production of hydrogen and methane from potato waste using a two-stage anaerobic digestion process. Bioresour. Technol. 99: 5078–5084. http://www.ncbi.nlm.nih.gov/pubmed/17964140 (Accessed February 18, 2015).

Annex 1. Startup period for each organic waste

CW:

Figure A1 showed the start-up phase in the bioreactor fed with CW. After a lag phase of approximately 30 days (start-up period), the bioreactor reached a stable methane production up to 40%. During the start-up period the CW has been added twice per week (on Tuesdays and Thursdays) in a diluted form (1:4) in order to let the microbial community develop and specialize to the substrate (Figure A1A). During the start-up phase, the flow cytometer cellular profiles, show a specification of the active biomass as showed in figure A1B.

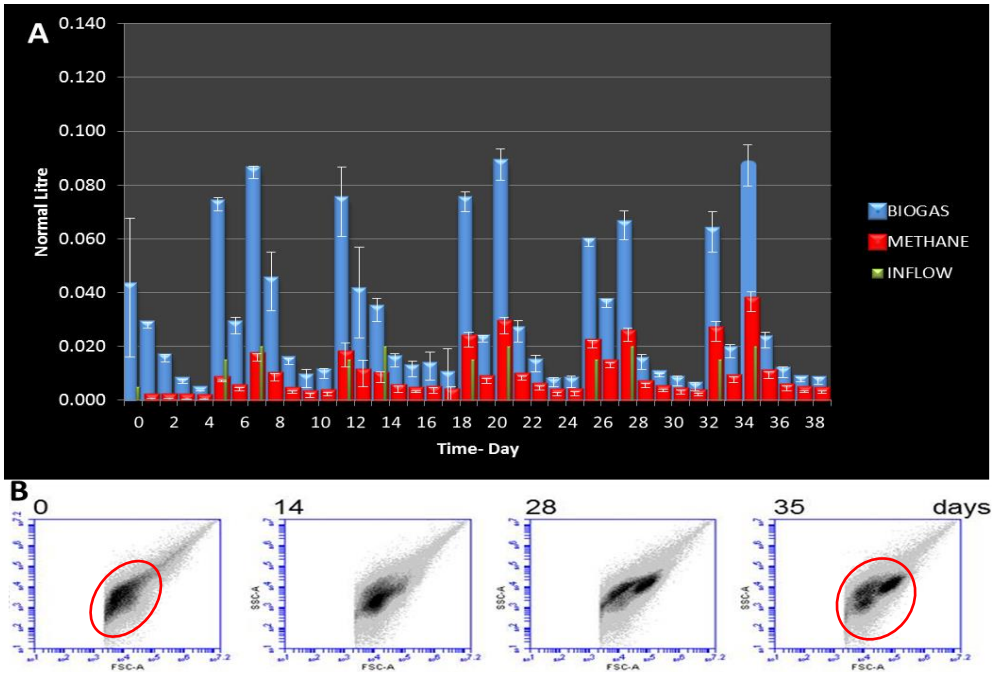


Figure A1. Start-up process in the bioreactor fed with CW

A. biogas (blue) and methane (red) production in the bioreactor fed with CW (inflow: green).

B. Flow cytometry cellular profiles plotted taking into account the cellular size (FSC - x axis) and the cellular complexity (SSC – y axis) during the experiment.

FWW:

Similar to what observed with CW, after a lag phase of approximately 30 days (start-up period), the bioreactor reached a stable methane production up to 50%. During the start-up period the FWW has been added twice per week (on Tuesdays and Thursdays) in a diluted form (1:10) in order to let develop and specialize the microbial community to the substrate (Figure A2).

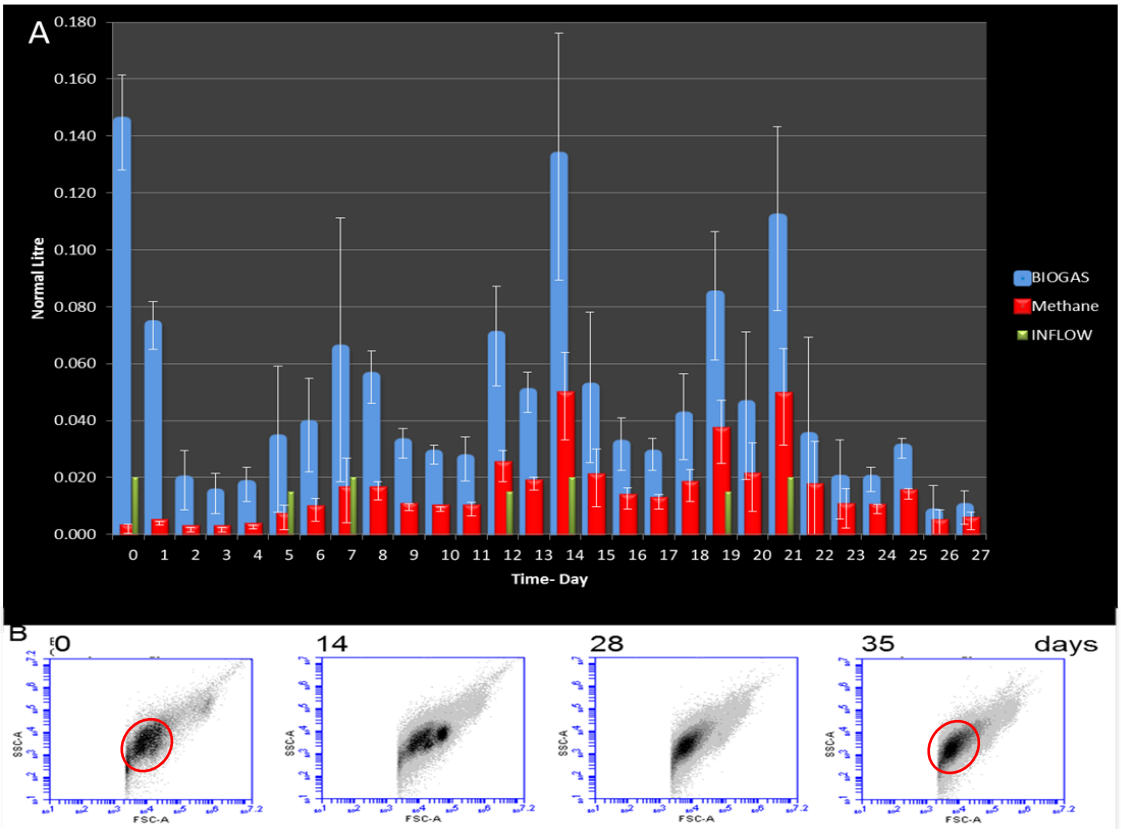


Figure A2. Start-up process in the bioreactor feed with FWW

A. biogas (blue) and methane (red) production in the bioreactor fed with FWW (inflow: green).

B. Flow cytometry cellular profiles plotted taking into account the cellular size (FSC - x axis) and the cellular complexity (SSC – y axis) during the experiment.

SK:

During the start-up phase the inoculum is acclimatized with the SK, inserted two times for week (on Tuesdays and Thursdays), this phase takes 21 days until the methane production reaches up to 40% (Figure A3A). During the start-up phase FC cellular profiles, show a specification of the active biomass as showed in figure A3B.

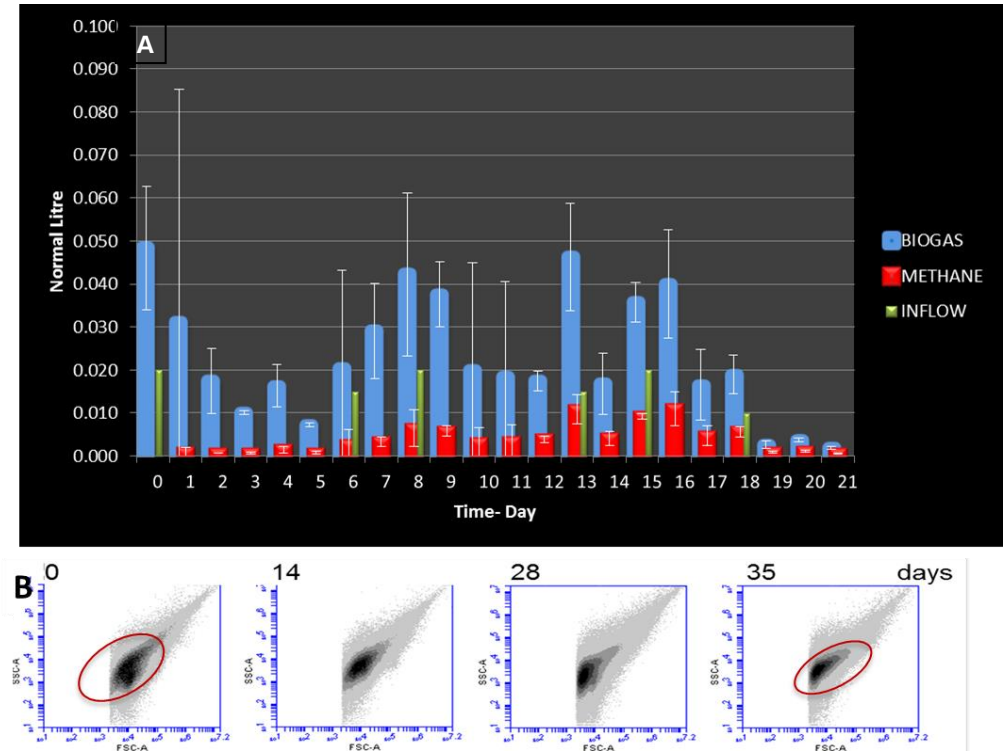


Figure A3. Start-up process in the bioreactor feed with SK

A. biogas (blue) and methane (red) production in the bioreactor fed with SK (inflow: green).

B. Flow cytometry cellular profiles plotted taking into account the cellular size (FSC - x axis) and the cellular complexity (SSC – y axis) during the experiment.

Annex 2. Hydrogen sulfide criticalities

The dispose of FWW pointed out an important problem in AD, the hydrogen sulfide (H_2S). In the presence of SO_4^{2-}, sulfate-reducing bacteria (SRB) compete normally with fermentative bacteria, acetogens and methanogens for same substrates. The SRB use SO_4^{2-} as an electron acceptor during anaerobic respiration (equation E1), while they use electron donors such as fatty acids, acetate or H_2, resulting in hydrogen sulfide (H_2S) as the final product (Dar et al. 2008; Stams et al. 2005; Rabus et al. 2013). The consumption of acetate is not the only problem provided by SRB, effectively their metabolic activity produce the most reduced form of sulfur (Barton & Fauque 2009), which is toxic for most of organisms and dangerous for iron structures (Enning & Garrelfs 2014).

$$3SO_4^{2-} + 2C_3H_6O_3 \rightarrow 3H_2S + 6HCO_3^- \quad \text{(E1)}$$

Moreover, some SRB were shown to play a role in the anaerobic oxidation of methane (AOM) in marine sediments (equation E2), normally in a syntrophic consortium with a methanotrophic archaea (Knittel & Boetius 2009). At the present day there is any proof for CH_4 consumption during the process of biogas production, but this is a quite new topic of research that should be taken in account for future deep analysis.

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad \text{(E2)}$$

The acidification of the pH is not enough to prevent the SRB growth because they can survive also at acid pH (Reis et al. 1990). Anyway, the metabolism of SRB is inhibited by the molybdate (MoO_4^{2-}), which is a structural analog of sulfate and inhibits the respiration of SRB (de Jesus et al. 2015; Biswas et al. 2009). The production of H_2S from the FWW substrate was showed in figure 11, in samples with (triangle) and without (square) a final supplement of 5 mM of molybtate. In the experiment without ad of molybdate the concentration of hydrogen sulfide increase very rapidly passing from 133 to 1250 µmol/l in 24 hours. In less than a week the concentration of H_2S reach 1980 µmol/l corresponding to approximately 64 mg/l of hydrogen sulfide, that is higher compared to the limit inhibiting concentration allowing the growth of acetogens bacteria (Chen et al. 2008). In the other hand, the ad of 5 mM of molybdate showed a small increase of hydrogen sulfide from 59 to 360 µmol/l after the first 24 hours, that correspond to approximately 4 fold less compared to the molybdate free analysis (1250 µmol/l). Interesting observe that after a week of incubation the concentration of H_2S was not increase but inversely it decrease to 210 µmol/l. The reduction of the H_2S concentration coincide with a 3 fold rise of the total cell number (Figure A4, grey triangle). Probably other microorganisms take advantage against the SRB population inhibited by the presence of molybdate. Instead, without Molybdate the total cell number (Figure A4, light green square) increase at the beginning and then remain stable along the week counting about the half of cells compared to the (+) Mo incubation.

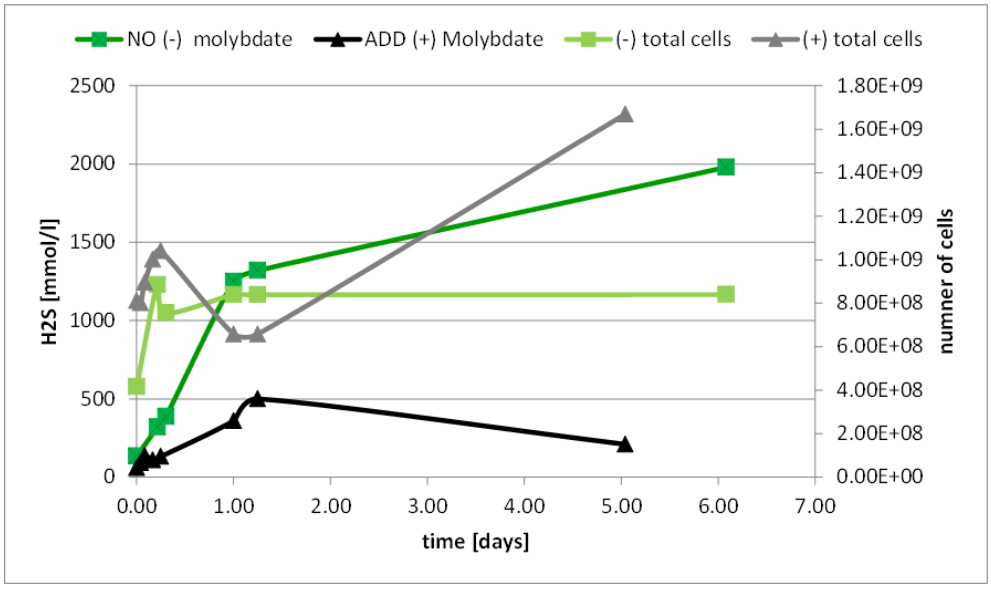


Figure A4. Molybdate SRB inhibition test.

This graph compare the production of hydrogen sulfide (H_2S) using FWW (Gnosis) as substrate. The dark green square (no Molybdate) and black triangle (+ 5 mM of molybdate) showed the production rate of H_2S after the ad of FWW substrate. The light green square and gray triangle the corresponding total cells number for samples without Molybdate and with 5 mM of molybdate, respectively.

Hypothetically, starting from the initial H_2S value of 133 µmol/l is possible to estimate from equation 1 how much acetate was “steeled” by the SRB per day, approximately 176 µmol or 5.7 mg of acetate. The maximal daily production of acetate measured before was equal to 57 mg corresponding to a 10 % acetate loosed every day.

This problem is quite common in AD process and should be treated more in detail in further project.

Annex 3. Solubility criticalities

To outcome the solubility problems due to the hydrophobic triglycerides mostly composing the SK, two tests were quickly evaluated in the following annex 2:

The use of concentrated HCl to break down the glycerol~fatty acids bonds

The increase of the temperature from 37 to 56°C to enhance the liquid form of the SK.

The use of HCl showed a clear improvement of the SK solubility in water. Once the pH in the SK solution was brought to around 4.0, the appearance of the sample completely changed going from a heterogeneous particulate to a limpid aqueous aspect (data not showed). The resulting aqueous sample was daily added starting with low COD concentration of 0,011 g/d (OLR = 0.55 kgCOD/m^{3}d) similar to the experiment showed in “Task 1.2.2., SK”. But after approximately 2 weeks the pH in the sample dropped to 3.0 – 3.5 inhibiting the most of microorganisms (data not showed), excluding the expected production of acetate. The pH of the aqueous SK solution had to be neutralized before further use.

On the other hand, the increase of the temperature to 56°C increased also the production of acetate of about 10 fold (figure A5) compared to the production at 37°C showed in the “Task 1.2.2., SK”. This experiment showed that in laboratory conditions with small volumes (20 ml) the maximal acetate production for SK at 56°C is 0.064 g of acetate every g of COD (OLR = 0.515 kgCOD/m^{3}d).

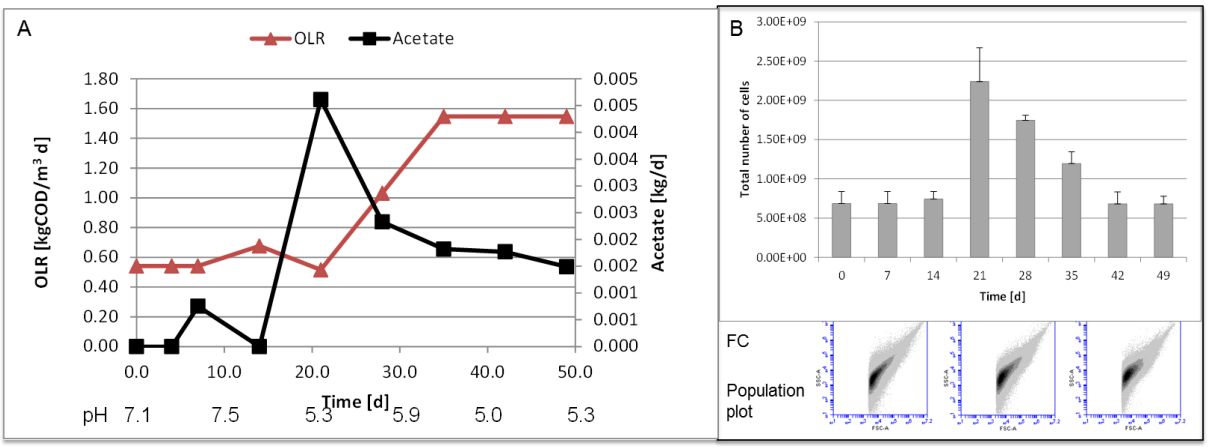


Figure A5. Optimization of acetate production from SK at 56°C.

A) This graph put in evidence the concentration of acetate (black square, kg/d) and the OLR (red triangle, kgCOD/m^3 d) in function of the time (days). In the bottom of the figure is also showed the value of the pH in the sample measured every 10 days.

B) Flow cytometry results, the upper graph showed the total number of cells (grey bars) along the experiment, and the bottom part showed a plot of the cellular complexity (SSC) and size (FSC) of the microbial community at the beginning, in the middle and at the end of the analysis.