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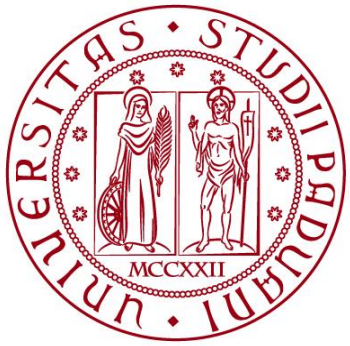

TESI DI LAUREA

Laboratory studies on the preservation of inocula for BMP testing

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ANNO ACCADEMICO 2021-2022

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Abbreviations

TS Total solids

WWTP Wastewater treatment plant

WWT wastewater treatment

VS Volatile solids

AD Anaerobic digestion

VFA Volatile fatty acids

BAm Basal anaerobic medium

MBRs Minibioreactors

BMP biochemical methane potential

LCFAs Long chain fatty acids

CSTR Continuously stirred tank reactor

GC Gas chromatograph

PPCO Polypropylen Copolymer

FEP Teflon

ISR Inoculum to substrate ratio

FDA Fluorescein Diacetate

PI Propidium Iodide

PBS Phosphate-buffered saline

FIRST PART

1. Introduction

The current energy crisis and the desired gas independence of European countries open new possibilities for the use of residual organic waste for biogas. Renewable energy generation using biogas and extended gas treatment (e.g., methanation) also decreases the use of fossil fuels and contributes to the reduction of climate-damaging emissions. The renewable alternative to gas from underground basins is represented by biogas from anaerobic digestion, as it is produced from organic waste treatment and can be used for heat and power generation. Even if this process is promising, nowadays the technology is not cost-effective enough to be competitive with natural gas, so the production to supply a large-scale population is still not possible. In this frame, biochemical methane potential (BMP) tests are fundamental to find new substrates, to analyse the factors influencing the process and to assess the efficiency of anaerobic digesters. The results obtained must be objective and ensure inter laboratory repeatability, so standardized procedures are established. Several factors such as the conditions and duration of these batch tests are strictly regulated by the Verein Deutscher Ingenieure, 2016 (Verein Deutscher Ingenieure e.V., 2016) and other studies (Holliger et al., 2016)(Angelidaki et al., 2009). However, the microbial inoculate for these measurements remains non-standardized as it has different sources depending on the location of the facility where the test is conducted. Usually, inoculum from local or regional wastewater treatment plants is used. Therefore, each tested microbial community has different specifics, and the results cannot be precisely compared. A possibility to overcome this problem is through the development of a standardized inoculum consisting of a stable microbial community which can be supplied to researchers. Initial investigations were carried out by Heerenklage et al., 2017 and Bhattad et al.,2016 by means of freeze-drying of the inocula.

The present work focuses on the preparation of standardized inoculum for distribution. The main strategies evaluated are freezing at -20°C with addition of DMSO as cryoprotectant and utilization of agar gel as carrier stored at 20°C. The time considered for the preservation is 14 days. The performances were estimated considering the quantity and quality of biogas produced during the BMP test after the storage time.

The Hamburg University of Technology (TUHH) and the Padova University (UniPD) worked together under the supervision of Prof. Roberto Raga, Ing. Jörn Heerenklage and Prof. Laura Treu to carry out the present work.

1.1. Renewable energy

Over the last 100 years global population more than quadrupled: the United Nations projects that it is going to increase from 7.7 billion in 2019 to 10.9 billion by the end of the century (Max Roser, 2019). In parallel to this trend, it is the energy demand. In 2020, oil and petroleum products contributed to 34.5 % of the gross available energy in Europe, followed by natural gas (23.7 %), whereas solid fossil fuels represented 10.2 %. In other words, 68.4 % of all energy in the EU was produced from coal, crude oil and natural gas (Eurostat, 2020). In this frame, it turns out to be crucial enhancing the recovery of materials, nutrients, and energy, trying to close the loop of production and disposal.

Considering biomass, anaerobic digestion plays a key role as it allows to recover biogas from wastes such as animal manure, human sewage, or food waste. The raw produced biogas typically consists of methane (50–75%), carbon dioxide (25–50%), and smaller amounts of nitrogen (2–8%). Trace levels of hydrogen sulphide, ammonia, hydrogen, and various volatile organic compounds are also present depending on the feedstock (Li et al., 2019). Raw biogas can be cleaned to yield purified methane (biomethane) that can be readily incorporated into natural gas pipelines making it renewable energy source (Holmes & Smith, 2016).

Even if this production pathway is well known, it doesn't satisfy the increasing demand of gas. In 2019 in Europe almost three quarters of imports of natural gas came from underground stocks from Russia (41 %), Norway (16 %), Algeria (8 %) and Qatar (5 %), causing not only environmental but also economic and political issues. The research in this field needs to go further and overcome the present limits of the process.

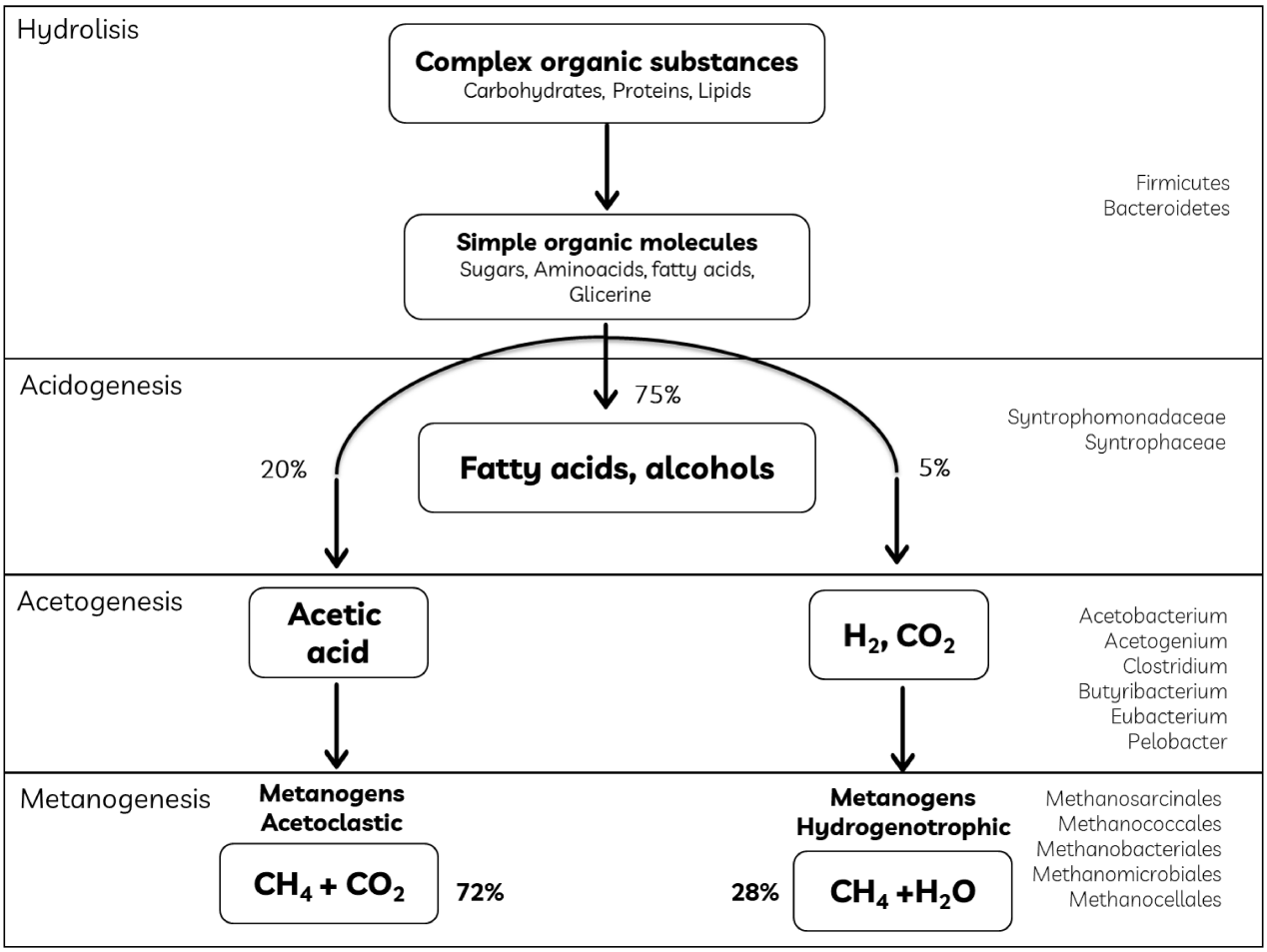
1.2. Anaerobic digestion

As mentioned before, anaerobic digestion represents the process in which organic matters are converted into biogas. The quantity and quality of biogas is function of many factors, as feedstock composition, digester retention time and temperature. Bacteria are involved in the first three steps, and archaea responsible for the final stage (Campanaro et al. 2018).

The AD reactors can work with many types of waste as input: sewage sludge, animal waste as pig manure or animal carcasses, organic municipal and household waste, agricultural waste as stem and crops, industrial waste as food/beverage processing waste and dairy wastes.
All these organic substances are suitable for the process and can be classified according to three macronutrients: carbohydrates, lipids, and proteins.

Carbohydrate substrates are represented mainly by lignocellulosic substrates that come from energy crops, by-products, and grass cuts. Through hydrolyzation carbohydrates are converted into polysaccharides, oligosaccharides and monosaccharides.
If the waste come from food processing, slaughterhouse, olive oil mill etc. lipids are highly present in the feedstock. As reported in table 2, fats may represent a valuable substrate for AD, on the contrary they are particularly dangerous when released in natural water basins, as they create a thin film over water that make it impossible for oxygen to dissolve and cause the death of water animals and plants. In Mediterranean countries, the annual amount of olive mill wastewaters is estimated to be up to 30million m^3 and a middle-scale olive mill is producing ~8m^3 wastewater per day (Treu, 2021).
In feedstocks coming from cheese and fish factories, pig, and poultry manure, we have significant quantity of proteinaceous substrates. It is important to notice that protein degradation leads to high ammonia concentrations which is the most common inhibitor of the AD process.

Table 1 *Scheme of anaerobic degradation and methane production (Maria Cristina Lavagnolo, 2020)*



The process take place in mainly four steps named hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Ponsá et al., 2008).

Biomass is made of long organic polymers that to be degraded must be broken into smaller constituents easily available to microorganisms. This process called hydrolysis is carried out by hydrolytic bacteria as *Firmicutes* and *Bacteroidetes* (Yan, 2021). They can convert high-molecular-weight polymeric components (carbohydrates, proteins, and lipids) into soluble organic substance (simple sugars, aminoacids, and fatty acids). These bacteria families resulted more robust to environmental stress, as ammonia presence and temperature changes, than the methanogens (Dimock & Morgenroth, 2006).

Contextually the process of Acidogenesis takes place and led to a further breakdown of the soluble organic compounds. Here fermentative bacteria groups as *Syntrophomonadaceae* and *Syntrophaceae* degrade organic monomers and oligomers, sugar, LCFA, amminoacids, producing VFAs and alcohols (Gujer & Zehnder, 1983). Generally, the biodegradation rate of LCFA is slower than the

hydrolysis process, which causes their quick accumulation in the AD system and may bring to inhibition of the process (Yan, 2021).

Starting from the substrates formed during the hydrolysis and acidification phase, the acetogenic bacteria produce acetic acid, formic acid, CO_2 and H_2. Hansen et al. (1998) reported that two different mechanisms must be considered depending on whether degradation occurs from LCFA or VFA. *Acetobacterium, Acetogenium, Clostridium*, *Butyribacterium*, *Eubacterium* and *Pelobacter* are the bacteria families responsible for this process (Ziels et al., 2017).

Methanogenesis represent the last step of the trophic anaerobic chain. Methane and carbon dioxide are indeed the only not reactive substances of the whole process, so they can be considered the last products. The production of methane can take place through two different reaction routes: one pathway involves methanogenesis by hydrogenotrophic bacteria, which operate the oxidation of hydrogen producing 28% of the final biogas volume. The second way is called Acetoclastic and involves the anaerobic conversion of acetic acid with the formation of methane and carbon dioxide. Most methane production takes place through this second mechanism, about 72%. The bacterias involved in this last step are *Methanosarcinales, Methanococcales, Methanobacteriales, Methanomicrobiales* and *Methanocellales* (Yan, 2021).

1.2.1. Process parameters

There are several key parameters that lead the AD process and must be monitored to have ideal operational conditions and avoid inhibition. The oxygen content is for sure one of the most important. It's relevant to point out that even a constant but small stream of air does not completely inhibit the process as species of hydrolysing, acidificating and acetogenic bacteria that are facultative anaerobes may be present. In any case, it's necessary to avoid the exposure of inoculum to ambient air as much as possible.

Another leading parameter is the substrate structure, so high reduction of particles size leads to larger surfaces where microbial can attach and increase the availability of substrate. Reported in **Figure 1**, there's an example of the difference between the biogas yield get from hackled and untreated hay used as substrate.

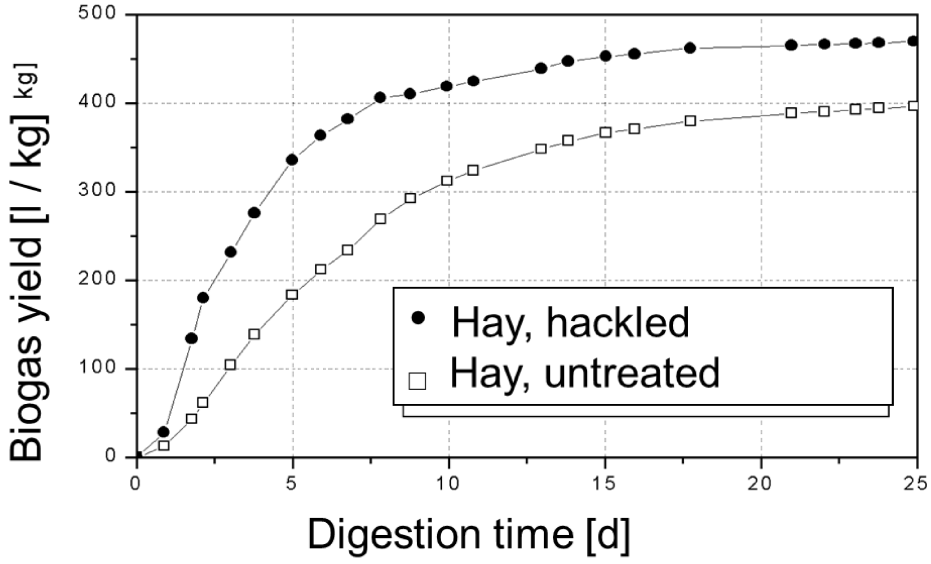


Figure 1 *Comparison between biogas yield from hackled and untreated hay (Maria Cristina Lavagnolo, 2020)*

Temperature as well has a big influence on the process. Usually thermophilic and mesophilic microorganisms are those most present. The optimum temperature range for thermophilic bacteria is 33 - 45 °C, those are applied for the treatment of household waste, industrial waste, and animal slurry with a high content of ammonia. For mesophilic bacteria the best temperature is between 50 – 60 °C and they are used for high yielding processes, often with advanced process technology and where sanitation is required.

PH should always be in 6.6 – 8 range, as it creates the ideal condition for all the bacterial families.

Finally, there are many inhibitory compounds that at certain concentration cause breakdown of the biogas process. Ammonium ion (NH_4^+) and free ammonia (NH_3) are the most important causes of inhibition; especially NH_3 can diffuse passively into the cell, causing proton imbalance and/or potassium deficiency (Kugelman & Chin, 1971). The methanogens are the most sensitive to this compound (Kayhanian, 1994).

Sulafate is widely present in many industrial wastewaters, and in anaerobic reactors it's reduced to sulfide. This reduction may lead to two types of inhibition: the first is due to the competition for substrates between methanogenic bacterias and sulfate reducing bacteria which suppress methane reduction, the second results from the toxicity of sulfide to various bacteria groups (Chen et al., 2008)

Other dangerous compounds are light metals (Na, K, Mg, Ca, and Al) as they lead to bacterial cell dehydration due to osmotic pressure. These elements can stimulate microbial growth if present in moderate quantities but in higher concentration cause toxicity (Soto et al., 1993).

Heavy metals can be present in significant concentrations in municipal sewage and sludge. The heavy metals identified to be of particular concern include chromium, iron, cobalt, copper, zinc, cadmium, and nickel (Jin & Bhattacharya, 1996). The main issue related to these compounds is that they are not biodegradable and can accumulate in the system to toxic concentrations.

Finally, a wide range of organic compounds can inhibit anaerobic processes. Organic chemicals which are poorly soluble in water may accumulate to high levels in the digesters. The accumulation of apolar pollutants in bacterial membranes causes the membrane to swell and leak, disrupting ion gradients and eventually causing cell lysis (Chen et al., 2008).

1.3. Biomethane potential test

1.3.1. Fundamental principle

Biochemical methane potential (BMP) tests are important to find new, efficient substrates and optimize existing processes. The BMP of a specific substrate defines the maximum amount of methane that can be produced by anaerobic digestion. This parameter has key importance in substrate characterization and efficient evaluation of anaerobic digestion plants. It is crucial for assessing the quality and monetary value of different substrate types and enables reliable comparison between AD efficiency.

Two types of analysis must be carried out when performing a BMP test: the blank assay, in which only inoculum is incubated; and the reference samples, containing the same quantity of inoculum with the addition of substrate. Both these investigations are important as the difference between the methane production from the references and those from the blanks give the quantity of methane produced by the only degradation of substrate as shown graphically in **Figure 2**. Another compulsory test for validation BMP is the positive control. It consists of a primary BMP test carried out using a standard substrate. This step is necessary to validate the inoculum activity with the substrate and

compare its performances with the well-known nominal value. Usually microcrystalline cellulose (CAS 9004-34-6) is used as standard substrate.

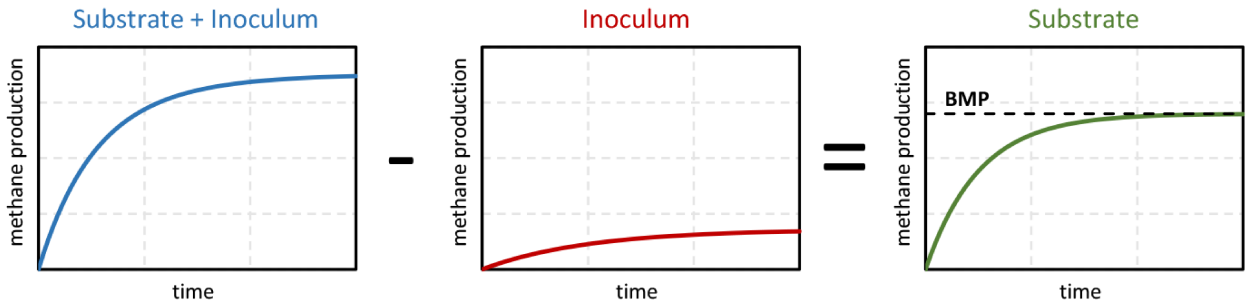


Figure 2 *Basic principle to calculate the methane production of the substrate (Koch et al., 2020)*

Reported in table 2 there are some examples of different substrates and the relative theoretical biogas potential. It is clearly noticeable that fats are the nutritional category with higher potential.

Table 2 *Theoretical biogas production (Maria Cristina Lavagnolo, 2020)*

Component	**Chemical formula**	**Theoretical bigas potential [Nm CH_4/tons VS]**
Carbohydrates	$(C_6H_{10}O_5)_n$	415
Sugar (glucose)	$C_6H_{12}O_6$	373
Starch	$C_5H_5O(OH)_2OCH_2OH$	415
Cellulose	$C_5H_5O(OH)_2OCH_2OH$	415
Fibres	C5H5O(OH)2OCH2OH	415
Fat	$C_{57}H_{104}O_6$	1014
Fat 1 (Lauric fatty acid)	$CH_3(CH_2)_{10}COOH$	952
Fat 2 (Arachidic fatty acid)	$CH_3(CH_2)_{18}COOH$	1041
Protein	$C_5H_7NO_2$	496
Protein 1	$C_5H_{11}NO_2$	574
Protein 2	$C_6H_{14}N_3O_2$	438
Protein 3	$C_4H_{10}N_2O_3$	293

Considering the standardised protocols proposed by Holliger et al in 2016, Holliger et al., 2021 for the validation of BMP tests, the results are acceptable if the following elements are fulfilled:

- it is necessary to perform simultaneously the BMP of the substrate, the positive control and the blank assay;
- the duration of the BMP tests should not be fixed in advance, and tests should only be terminated when daily methane production during three consecutive days is <1% of the

accumulated volume of methane. Also, gas composition by means of Gas Chromatography (GC) is taken at regular intervals (Koch et al., 2020);

- the BMP results should be converted in standards condition of temperature and pressure (273.15 K and 101.33 kPa) per mass of volatile solids (VS) added, with the unit Nml CH_4/gVS;
- the BMP of the substrate and the positive control are determined by subtracting the methane production of the blanks from the gross methane production of the references;
- the results must be rejected if the BMP of the positive control is <85% and >100% of the theoretical BMP (for cellulose: <340 and >390 $NmLCH_4$/gVS).

During the metabolism of the substrate in the BMP tests, the typical net biogas formation curves shown in **Figure 3** are plotted. They resulted from the difference between the biogas volume that is produced by converting the substrate and the biogas volume that arises from the inoculum's own production.

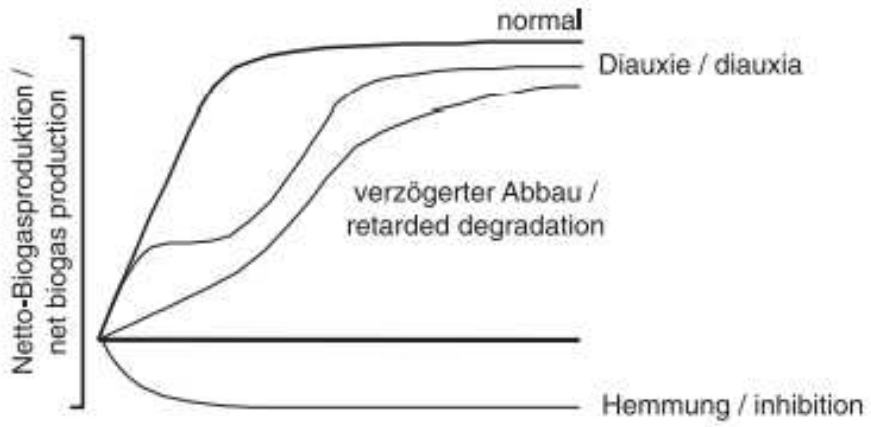


Figure 3 *Typical biogas formation curves in BMP tests (Verein Deutscher Ingenieure e.V., 2016)*

The Normal curve shows a limited exponential growth because of fast substrate conversion, whereas substrates that degrade with difficulty show delayed/retarded degradation type of curve. The Diauxic curve describes the exponential increase of biogas quantity after a certain time, thereby indicating a lag in the time to reach a rise in biogas production. This is denoted as "Lag Phase." The final one, Inhibition, takes place when the sample without substrate produces more biogas than the one with substrate.

1.3.2. Inoculum and substrate

In this chapter, some suggestions about inoculum characteristics are reported from literature.

The inoculum should be taken from an active anaerobic digester working at steady state conditions. Therefore, it includes a large variety of microbial communities able to digest different types of organic substrates. If the reactor has very simple feed composition, different inoculum should be mixed. The inoculum should be preserved at the temperature that it was inside the reactor, either mesophilic or thermophilic inoculate can be used. Before the BMP test, the endogenous methane production should be detected: the methane produced from the blanks samples should be below 20% of total methane production (reference samples). If from the blanks a high biogas production is detected, the inoculum should be "degassed", meaning pre-incubated for 2-5 days in order to deplete the residual biodegradable organic material present in it. Otherwise, it should be used as fresh as possible. If coarse material is present, sludge should be homogenised by sieving through a 1-5 mm mesh screen (Angelidaki et al., 2009) (Holliger et al., 2016).
As for the inoculum, substrate preparation should be minimal to avoid alteration of its properties and digestibility. The total solids (TS) and volatile solids (VS) should always be detected. About the quantity of substrate to add, it must respect the Inoculum to substrate ratio (ISR) defined according to Holliger et al., 2016.

$$ISR = \frac{VS\ inoculum}{VS\ substrate} \quad (1)$$

It important to ensure that the portion of VS coming from the inoculum is bigger than the one coming from the substrate to avoid acidification or inhibition problems. For this reason, ISR should be always greater than 1, and for most of the applications between 2 and 4.
Basal Anaerobic Medium is important when the involved substrate have deficiency of nutrients, micronutrients, and vitamins. That's the case of some solid substrates and energy crops (Lindorfer et al., 2007).

In the previous paragraph, between the guidelines to accept/refuse a BMP test, it was mentioned that the results must be rejected if the BMP of the positive control is <85% and >100% of the theoretical BMP (for cellulose: <340 and >395 $NmLCH_4/gVS$). This result comes from the composition of the substrate, indeed, the theoretical biomethane potential (CH_4 Nml/gVS) can be calculated considering the stoichiometric balance reported in the following formula:

$$C_aH_bO_c + \left(a - \frac{b}{4} - \frac{c}{2}\right) \cdot H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4}\right) \cdot CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4}\right) \cdot CO_2$$

(2)

Cellulose was used as carbon source in this work, the molecular formula is $C_{12}H_{20}O_{10}$ and the molar mass is 324.28 g/mol. For simplicity, methane and carbon dioxide are considered ideal gases. 1 mol of an ideal gas occupies a volume of 22.4 L under standard conditions (273.15 K and 1013.3 hPa). Thus, the theoretically maximum achievable net biogas volume of cellulose is 830 ml/g cellulose. With a methane content of 50% in the biogas, the methane potential is 415 mL/g cellulose (Tiemann, 2017). According to the VDI guideline, the biomass of an inoculum is considered active if 670 Nml/g cellulose net biogas and 335 NmL/g cellulose net methane are produced (Verein Deutscher Ingenieure e.V., 2016). This corresponds to about 80% of the theoretical potential.

1.4. Storage time and temperature influence

Heldal Hagen et al., 2015, investigated the influence of different storage temperatures and durations on the microbial composition and activity of the inoculum. The experimental approach took place under mesophilic conditions and with cellulose as reference substrate. Hagen chose anaerobic seeds sludge from a regional biogas plant as the inoculum, which was sieved and diluted with water. The samples were stored for up to eleven months at temperatures of -20°C, 4°C and room temperature. The BMP test after storage last for 40 days and was carried out at mesophilic conditions. The experiment was run in triplicate. Hagen also set up samples with fresh and untreated sludge to be able to compare them with the processed sludge. The following results (table 3) were taken from (Heldal Hagen et al., 2015).

Table 3 *Cumulative methane produced from the BMP test after storage of samples. Results coming from (Heldal Hagen et al., 2015)*

Storage period	Cumulative methane production from BMP test after storage [Nml/gVS]		
	Room temperature	4°C	-20°C
1 week	368	388	381
1 month	389	427	408
2 months	321	272	259
6 months	223	186	139
11 months	221	183	132

The BMP test with fresh and untreated inoculum resulted in a methane production of 372 Nml/gVS. It was found that samples stored for more than a month produced less methane than samples with the fresh reference inoculum. Moreover, the longer the samples were stored, the less was the cumulative methane volume per grams of volatile solids. The samples stored for less than a month at different temperatures reached higher gas volumes than the unprocessed inoculum. Also the methane content decreases with increasing of storage time. In the last five months, all the samples almost lost any activity. Samples stored for more than two months at a temperature of -20 °C achieved the lowest methane content compared to other storage temperatures. This resulted in the worst preservation method.

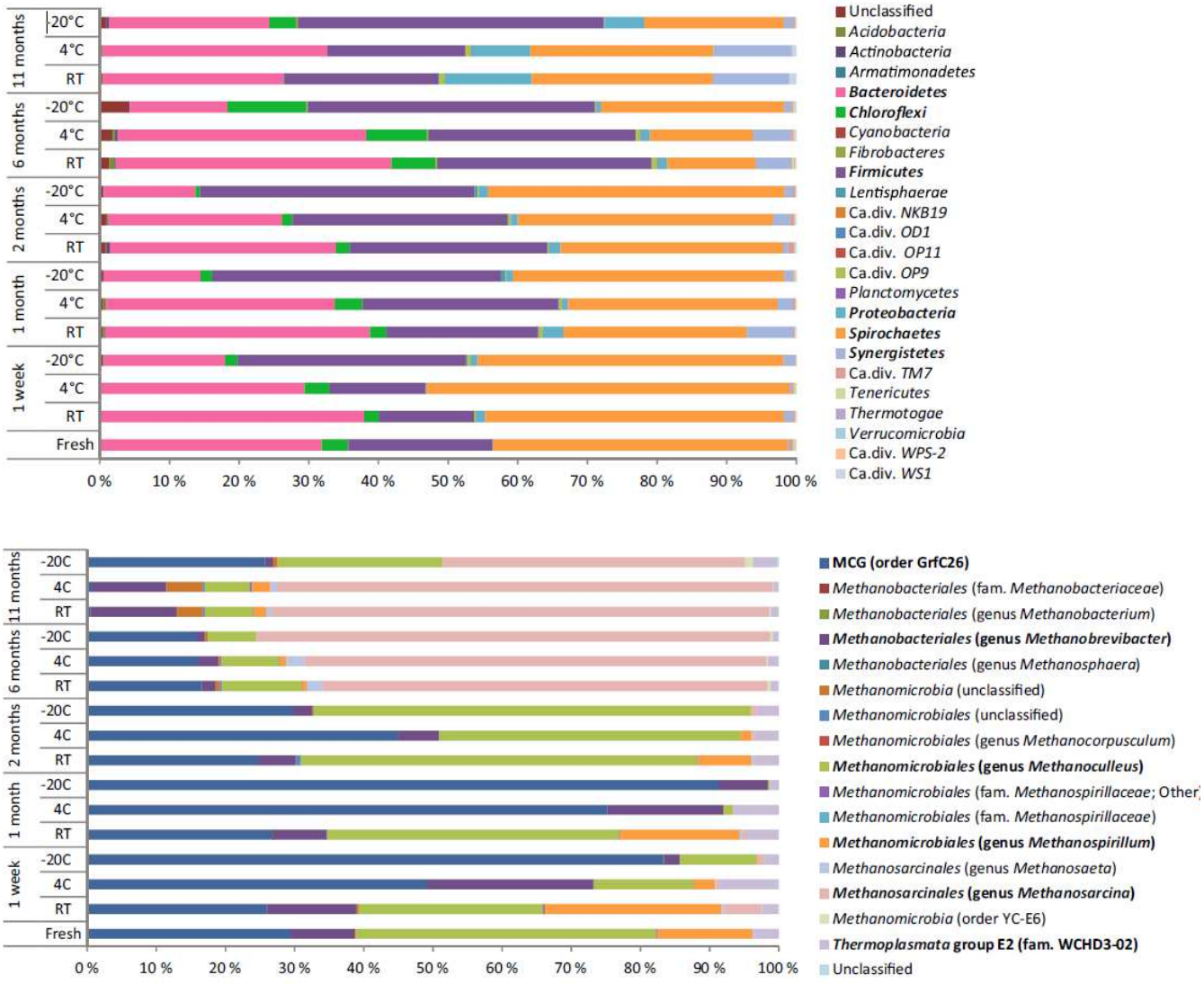


Figure 4 *Relative abundance [%] of respectively bacteria and archaea identified in batch digesters after anaerobe digestion of cellulose for 40 days at 37 °C, using an inoculum stored at room temperature, 4 or –20 °C for different periods of time.*

Regard as the results reported in **Figure 4**, the following considerations are stated. The proportion of methanogenic archaea of the species Methanoculleusis was about 40% in the untreated sludge. After a storage period of one month at a storage temperature of 4 °C, this species can only be found at 2%. The quanity of Methanoculleus increases up to 45% at room temperature. At a temperature of - 20 °C, these can no longer be detected after one month. After a storage period of two months, the proportion of these methanogens in all samples is more than 45%. The perentage of Methanoculleusis about 10% after a storage time of six months. The genus of Methanosarcina accounted for less than 1% in the unprocessed samples. The proportion of these increased drastically over the storage period. After six months preservation, Methanosarcina were detected at levels higher than 65% in the samples stored at room temperature. At 4 °C and - 20 °C the proportion was 75%. After eleven months, the proportion in the samples at room temperature increased to 70%. The proportions of the samples at 4 °C and -20 °C dropped to 70% and 40%.

These results illustrate that storage temperature and storage duration have significant effects on the microbial composition and activity of inoculum. However, the preservation strategy applied in this study is out of the question for further research since losses in methane production of up to 65% after about a year are not promising. The goal of producing a standardized inoculum that can also be stored over a longer period of years would not be met.

1.5. Preservation strategies

In this chapter, all the preservation strategies considered in this work are presented. Besides DMSO and agar gel, glycerol and gel rite are discussed too. These last two methods do not represent the main topic of this thesis, but they were applied in the pre-test reported in chapter *3.1 Pre-test on preservation strategies.*

Storage of anaerobic cultures involve usually periodic reinoculations into freshly prepared nutrient media and maintenance. This may have serious drawbacks, for example the possible loss of morphological, physiological, and biochemical signs by the microorganism, which results in spontaneous mutations (Bhattad et al., 2017). A valid option is freezing with the use of cryoprotectants. Frozen products are biologically inactive and therefore allow long term storage without loss of quality (Staab & Ely, 1987). As crystallization of cellular water occurs at low temperature, cryoprotectants are necessary to protect cells from the consequent damages. They fall into two main categories: intracellular and extracellular (Janz et al., 2012).

Intracellular cryoprotectants enter the cell and directly prevent damaging crystallization, acting as an antifreeze agent. Dimethyl Sulfoxide (DMSO) and Glycerol, two intracellular cryoprotectants, were selected for application in the present work.

Extracellular cryoprotectants do not enter the cell but help to stabilize the osmotic balance during the freezing process. As they do not enter the cell, they are easier to remove after use, but may contribute to a prolonged lag phase as they do not prevent ice crystals from forming within the cell.

DMSO is regarded as an all-purpose cryoprotectant, being generally effective and able to infiltrate rapidly into cells (Yu & Quinn, 1994)(Hubálek, 2003). However, depending on the concentration, it is toxic at room temperature (Hoefman et al., 2012). Therefore, also glycerol was considered in this study as it resulted to be less toxic than DMSO at 37°C (Soltys et al., 2012).

Glycerol has been widely investigated for the preservation of bacteria with good results (Doebbler, 1966) (Howard, 1956)(de Leeuw et al., 1993). From the studies of Bryukhanov and Netrusov (2006) it was shown that after long-term preservation (i.e. 4 years) the samples containing Methanogenic archea are efficiently preserved but must be reinoculated twice per month in order to get the initial growth rate.

Besides the good results in preservation performance, refrigeration and freezing may cause high expenses and difficulties when cultures need to be transported for large scale distribution.

Gel preservation was considered as it is easy to operate and efficiently keeps microbial activity at low cost. The strategy applied was shaped considering two studies of Yan et al. In the first one, 5 mL of methanogenic consortium was efficiently preserved for 168 days in agar gel at 24°C, (Yan et al., 2020). In the second, ammonia-stressed AD reactor was recovered by the addition of preserved bioaugmentation consortium in gel (Yan et al., 2021). This novel approach using gel seems to be promising as it creates a layer that reduces the detrimental effects from environment (Barbabietola et al., 2016) and the inhibition caused by ammonia (Banu et al., 2018). However, the gel could be used as a substrate for the inoculum and make difficult the long-term storage of the samples.

To have a more holistic view, beside agar gel also gelrite was investigated. It can be substituted for agar in many routine medium applications, and in some cases, it may give higher viable cell recoveries than similar media solidified with Agar (Shungu et al., 1983).

A pre-test was performed to understand which methods suits better the inoculum considered in this work, the experimental set up and the results are reported in paragraph *Pre-test on MBRs materials*.

2. Material and methods

This section describes the materials used, steps involved, and later the formulas applied in the calculations. The figures exhibit the essential materials that were utilized in the experiments.

2.1. Materials

2.1.1. Digestate inoculum

The inoculum for the preservation experiments was taken from the Köhlbrandhöft municipal sewage treatment plant in Hamburg. The first step of the WWT process is mechanical cleaning. In this way, coarse solids, sands, and gravel are removed. After that, the wastewater flows through various primary clarifiers. The remaining solids settle in the activated sludge tank, where the digestate was taken. Usually, this inoculum has a very high endogenous biogas production, so it is incubated from 1 to 3 weeks at 37°C to enhance the degradation of residual organic material.
Every time a new inoculum sample is taken from the reactor, TS and VS are analysed as there could be slight variations in its activity. In table 4, the characteristics of the inoculum sample used for the final experiment are reported. The formulas applied are reported in chapter *2.4.1. Chemical analysis.*

Table 4 *Characteristics of inoculum*

	TS [% WM]	VS [% DM]	PH	FOS/TAC
Sieved inoculum	1.912 ± 0.003	63.546 ± 0.049	7.8	0.197

In **Figure 5**, the sieved inoculum before and after centrifugation is represented. The sludge withdrawn from the mentioned WWTP usually has a TS around 2-3%. However, process parameters changes may occur and cause variations in the inoculum quality. In this case, centrifugation was helpful in increasing the concentration of active biomass and removing the liquid phase, that represents almost the 30% w/w of the fresh sludge, as was confirmed by further analyses.

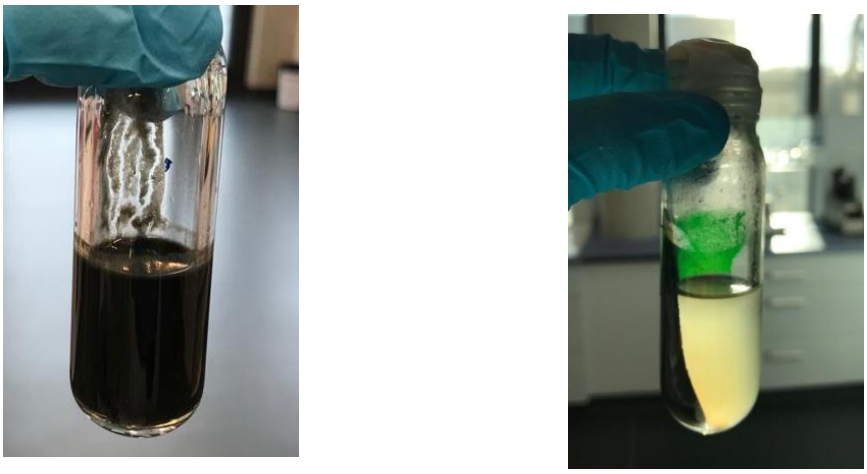


Figure 5 *Pictures representing on the left: sieved inoculum before centrifugation, on the right: sieved inoculum after centrifugation.*

2.1.2. Cellulose

As source of carbon cellulose was used; specifically, it was Microcrystalline cellulose by Avicel® PH-101 from Fluka analytical with an average particle diameter of 50µm. The sample of cellulose used as substrate is the same in all the present work. It was analysed for total solids (TS) and volatile solids (VS), the results are reported in table 5:

Table 5 *Characteristics of substrate*

	TS [% WM]	**VS [% DM]**
Cellulose	95.743 ± 0.107	99.936 ± 0.039

2.1.3. Basal anaerobic medium

The basal anaerobic medium was prepared according to the one proposed by Angelidaki et al., 2009. To 975 ml of distilled water the stock solutions reported in table 6 were added. After the preparation it was flushed with nitrogen to remove air residues and stored at 4°C.

Table 6 *Constituents and concentrations of the basal anaerobic medium (Angelidaki et al., 2009)*

Stock solution	**Component**	**Concentration in stock [g/L]**	**Volume in BAm [mL/L]**
A	NH4Cl NaCl MgCl2·6H2O CaCl2·2H2O	100 10 10 5	10
B	K2HPO4·3H2O	200	2
C	Resazurin	0.5	0.100

D (trace metals and selenite)	FeCl2·4H2O H3BO3 ZnCl2 CuCl2·2H2O MnCl2·4H2O (NH4)6Mo7O24·4H2O AlCl3 CoCl2·6H2O NiCl2·6H2O Ethylenediaminetetraacetate Concentrated HCl Na2SeO3·5H2O	0.05 0.05 0.05 0.038 0.05 0.05 0.05 0.05 0.092 0.5 1 0.1	1
E (vitamin mixture)	Biotin Folic acid Pyridoxine acid Riboflavin Thiamine Hydrochloride Cyanocobalamine Nicotinic acid P-aminobenzoic acid Lipoic acid DL-panthothenic acid	2 2 10 5 5 0.1 5 5 5 5	1

The equipment used for the BA medium is reported in **Figure 6**. The anaerobiosis bottle containing the medium has two entrances closed with two rubber septa. On one side it is continuously connected to a nitrogen bag, to enhance the liquid extraction and guarantee anaerobic conditions. On the other side, it can be pierced with the syringe for BA medium extraction.

Figure 6 *Apparatus for BA medium withdrawn in anaerobic conditions.*

2.2. Test set up

The equipment and the methodology used to measure the biogas production are described in this chapter. Notice that before the use of these apparatus is always necessary to check the possible presence of leakages in the system.

2.2.1. Minibioreactors

The minibioreactors (MBRs) can be used as small scale CSTRs for BMP testing. These apparatuses are composed of a centrifuge bottle with volume between 25-50 ml made of glass, Teflon (FEP) or Polypropylen-Copolymer (PPCO). A rubber septum located between the cap and the vessel allows to take gas samples through a needle, the septum should be thick enough to be pierced many times without losing its characteristics. A plastic tubular stiffener with a hole in the middle is put on the rubber septum to give it rigidity. Finally, a screw cap ensures the closure and the adherence between the septum and the vessel. All these components of the system are represented in **Figure 7**.

This equipment has many advantages, first represents a "all in one" test system as the vessels can be used as centrifuge bottle and for BMP test reactor so it's not necessary to shift from a container to another. In this way, the interaction of inoculum with atmospheric air is decreased and also the possible loss of material attached to the vessel surfaces. Secondly, the MBRs are easily handling, and

space saving thanks to their relatively small dimensions, so it is possible the set-up of experiments with many MBRs contextually or the analysis of different temperatures, moving the reactors to a different fridge/oven.

Before any experimental set up is necessary to check the tightness of the system. Therefore, 5 ml of water were poured inside each reactor. The apparatus was sealed and through a siring 10 ml of atmospheric air were injected piercing the rubber septum. The reactors were stored at ambient temperature for 24 h. The following day, the quantity of air still inside was measured using the instruments described in paragraph *2.3.2.Biogas volume.* Only the reactors still having 10 mL of air inside can be considered reliable and were used for the experiments.

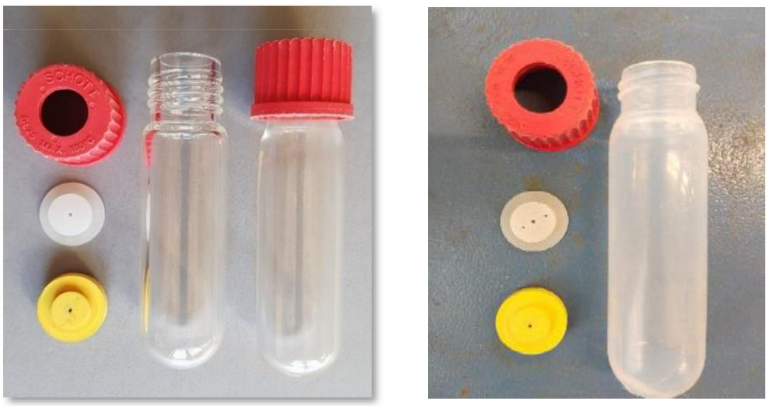


Figure 7 *On the left glass MBR components, on the right plastic MBR components.*

In table 7, the main different MBRs characteristics are reported from Thermo Fisher product specifications and Neubert Glas. The PPCO tubes and the FEP tubes are both models from Fisher Scientific Inc. The glass tubes belong to Neubert Glas. The glass tubes have a maximum load in the centrifuge of 4000 rpm In *Pre-test on MBRs materials,* a test to analyse the different performances of three MBRs materials is described.

Table 7 *Characteristics of different PPCO, FEP and glass MBRs (Thermo Fisher specifics).*

Model designation	Material	Acronym	Cold resistance [°C]	Volume [mL]
3119-0050	Polypropylen-Copolymer	PPCO	-40	43
3114-0050	Teflon	FEP	-100	46
0446-32-282100	Glass	Glass	-80	34

2.2.2. Eudiometers

Eudiometers were used to analyse high volumes of digestate, as up to 500 ml can be connected to the system. This apparatus is included in the manometric methods to measure biogas production in term of pressure variation. Therefore, it consists of a vertical 1L tube filled with a barrier solution made of NaCl/Citric acid, that avoid the diffusion of CO_2 in the liquid. The bottom of the tube is connected to a levelling bottle where liquid in excess can accumulate. On the top it is connected to the inoculum kept in a glass bottle. At the beginning of the test, the tube is filled of liquid and shows a biogas volume of zero; as the biogas volume increase, the pressure inside the system grows and the liquid solution is pushed in the levelling bottle, so the graduated tube shows the quantity of biogas currently produced. According to the VDI (Verein Deutscher Ingenieure e.V., 2016), the gas volume produced has to be read off when the levels of the confining liquid in the eudiometer tube and in the levelling bottle are the same. Hence, it is necessary to bring manually the levelling bottle to the same level of the liquid in the eudiometer in order to have an accurate value of the produced biogas.
Injecting a fixed amount of nitrogen in the sealed system the tightness must be proved. The following day, if the quantity of gas red on the eudiometer tube is the same as the day before, the system has no leakages and is ready to be used.

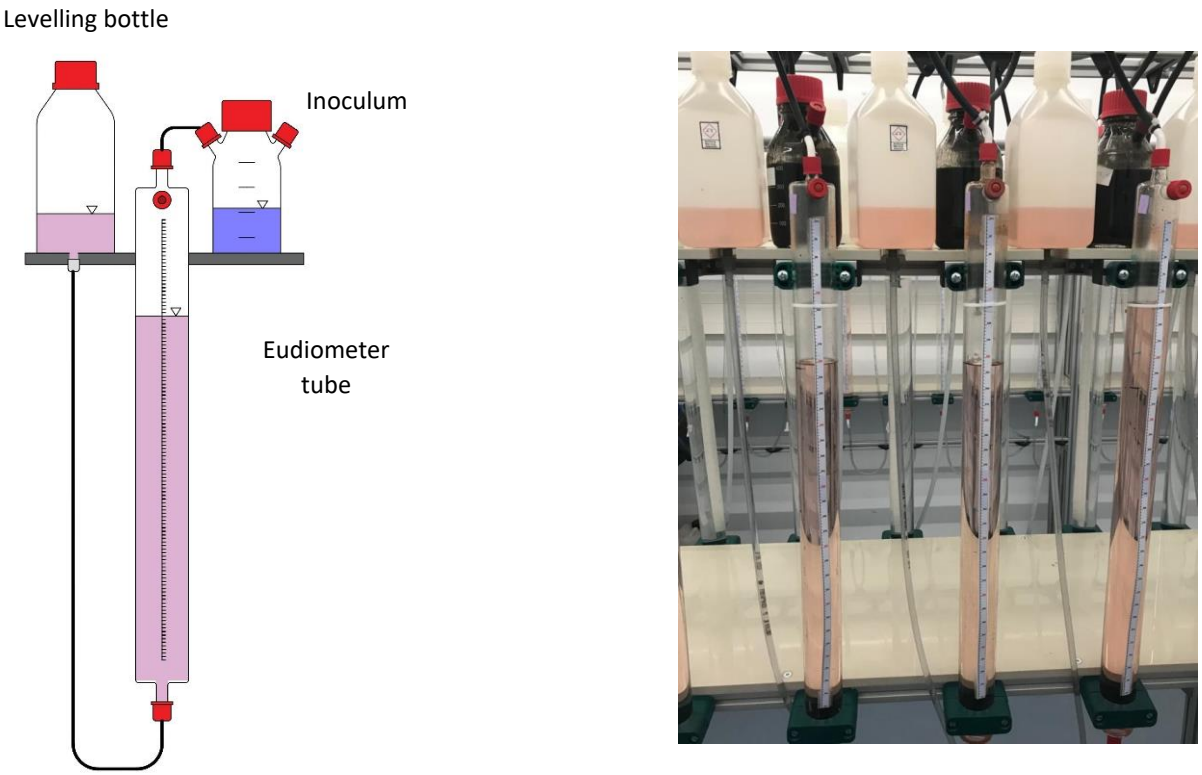


Figure 8 *Schematic representation and picture of apparatus for gas volume measurement with Eudiometer tube.*

2.3. Analysis

The inoculum characteristics were defined through the following chemical analyses: TS, VS, pH and FOS/TAC that are detailed in the following chapters.
The biogas production was evaluated considering two different analyses: the biogas volume and the variation of composition in time. All the analyses below are the same for both the pre-test and primary test involved in this experimental work.

2.3.1. TS, VS, PH, FOS/TAC

To assess the quality of inoculum the following analyses were carried out, the correspondent formulas are reported in *2.4.1 Chemical analysis*.
Total solids, volatile solids and pH were determined following the Standard DIN 12880 (Deutsches Institut für Normung e.V., 2001). The drying oven used was BINDER GmbH, type 18400300002030; the muffle furnace was M 110 der Heraeus Holding GmbH.
PH was measured at the beginning and at the end of the BMP test with the pH meter PH 3310. The expected value should range from 7 to 7.7.
In **Figure 9**, the pictures of these machineries are reported.

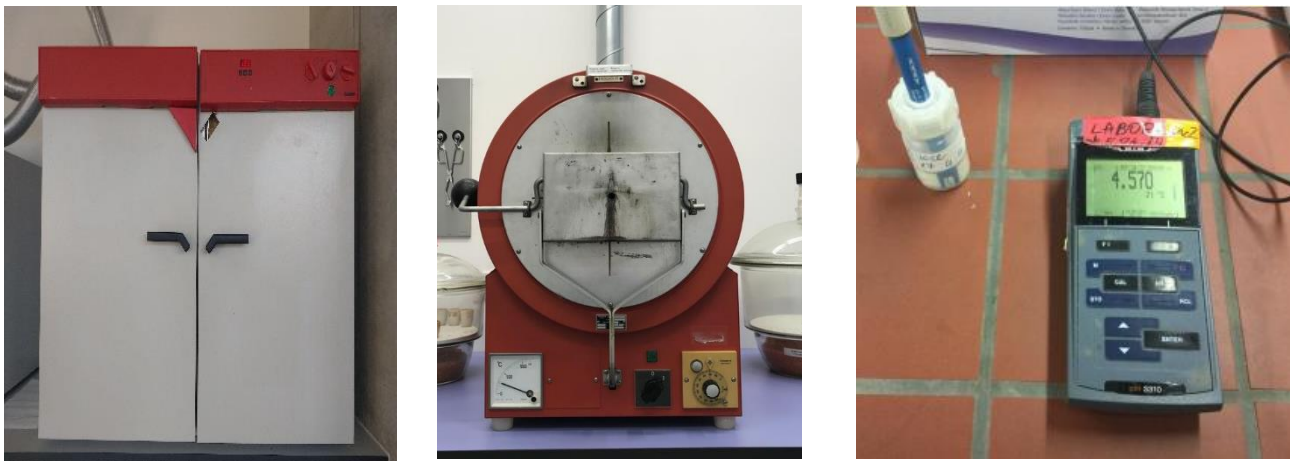


Figure 9 *Pictures representing from left to right: the drying oven, the muffle furnace, the pH meter.*

In some cases, knowledge of pH value is not enough to guarantee stability of the digestion process. If organic acids formed during anaerobic digestion accumulate, the methanogenic bacteria are inhibited. However, when a substrate with high buffering capacity is used, the accumulation will not

necessarily lead to a decrease of the pH value. For this reason, FOS/TAC value has been recognised as a guide parameter to assess fermentation processes and to recognise disturbances at early stages. It describes the ratio of volatile fatty acids (FOS, mg/L acetic acid equivalents) to the total inorganic carbonates (TAC, mg $CaCO_3$/L). The measurement is a titration test (Nordmann method) that has been adapted for the calculation of the ratio of acid concentration and buffering potential in the fermentation substrate. The method is based on the observation that during titration of bicarbonate solution with sulphuric acid, the typical pH drop is shifted from 5 to 3 if organic acids are present in the solution. For a stable process, a FOS/TAC value <0.3 is considered safe, although each plant has its own optimal ratio. This can only be determined by long-term observation and regular checks, as there is a strong dependence on the substrate.

This analysis was carried out following the guidelines proposed during the *Practical Course of aquatic chemistry* at TUHH, held by Kaltschmitt & Korner, 2021. The machinery used in this work was the automatic titration unit TIM 854, Hach-Lange GmbH.

2.3.2. Biogas volume

Considering the MBRs, it's not possible the direct reading on the instrument of the biogas volume, so the use of a specific tool was necessary (represented in **Figure 10**). The method is still based on the pressure developed inside the MBRs. It works through a needle connected to the top of a graduated glass pipette; the pipette is kept in a cylinder filled with a barrier solution and it has an opening on the bottom so that the liquid can enter the pipette. When needle pierces the rubber septum of the MBR, biogas accumulated in the reactor is driven into the pipette, where because of the increased pressure, the liquid level decrease and make it possible to read the detected volume of biogas. The barrier solution in the cylinder is a synthetic oil belonging to the group of polydimethyl siloxane, which name is Silox (Polydimethyl-Siloxane, Fa. Ritter, Germany). Using this oil instead of water bring many advantages: even if its viscosity is like water, it has less evaporation due to its lower vapour pressure, resulting in greater stability and in more consistent measurement result. It also shows in general, a lower chemical reactivity than water.

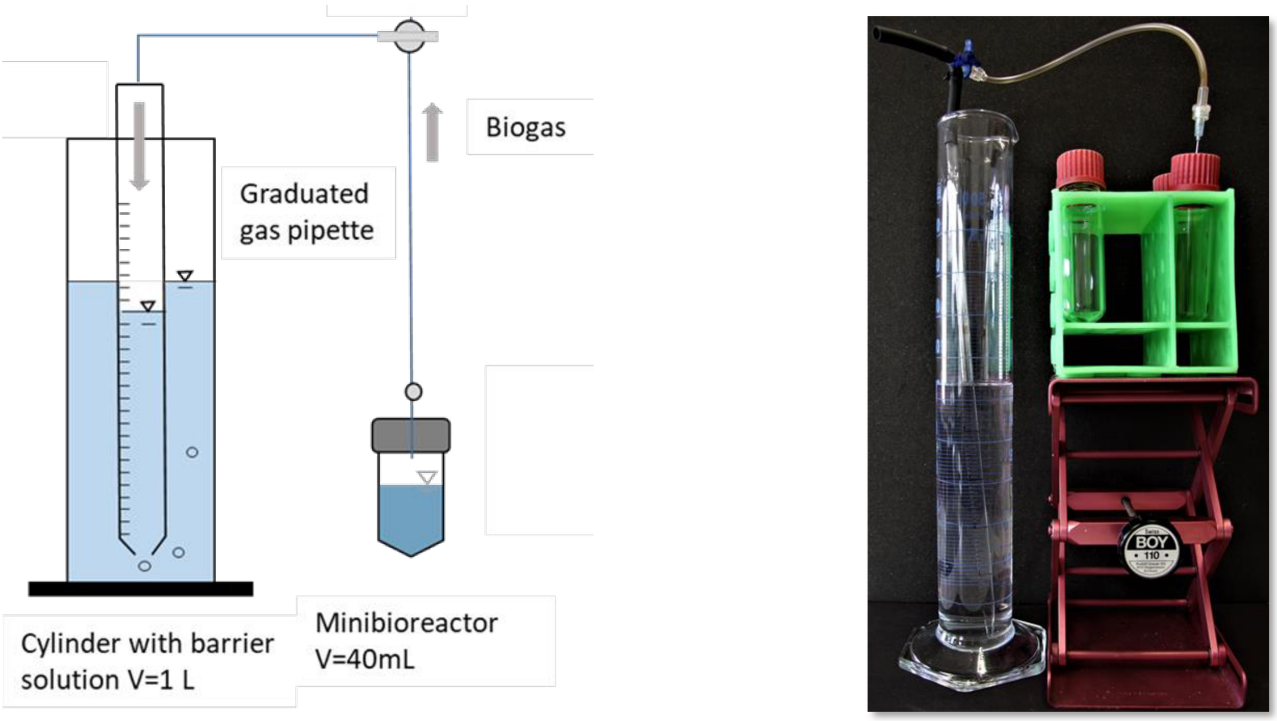


Figure 10 *Schematic representation and picture of the apparatus used to quantify the biogas produced in the MBRs.*

2.3.3. Biogas composition

The percentage of methane and carbon dioxide in the biogas were detected using a Gas Chromatograph, specifically the one used in this work is Agilent/HP 6890 GC. This analytical technique allows to separate the chemical components of the biogas in the headspace of the MBR and then determine how much each substance is present.

The first step to perform an analysis is to introduce a gas sample into the GC with a syringe piercing a rubber septum. Once the sample is injected, it is mixed with the so-called carrier gas and flows through the column inside the oven. The carrier gas represents the mobile phase, it moves through the column at a constant flow rate and has the only purpose to carry the sample through the system, hence an inert gas is applied, as helium or nitrogen. The column is where the separation of the gases composing the mixture takes place. It is a spiral pipe made of stainless steel or glass containing the stationary phase. The stationary phase is arranged as a packed column and retains the chemicals passing through. It can be made of a variety of materials, either liquids or solid. Depending on the type of gas, it interacts for more or less time with the stationary phase and this different retention time permits the separation of the gas from the mixture (Torres, 2016).

Trough the GC it was possible to get the precise percentage of methane, carbon dioxide, nitrogen, and oxygen in the headspace gas. It is necessary to rearrange the values to get the real percentages of methane and carbon dioxide in the biogas, as the results from the GC consider the composition of the

headspace gas. It was particularly important to monitor the changes in the composition of gas in time, so this analysis was repeated around three times per week. GC run needs 10 minutes each to be carried out, so for time limitations it was not possible to analyse all the samples every day. For this reason, linear interpolation was applied to calculate the values between GC measurements. The formula is described in paragraph *2.4.3 Methane production*.

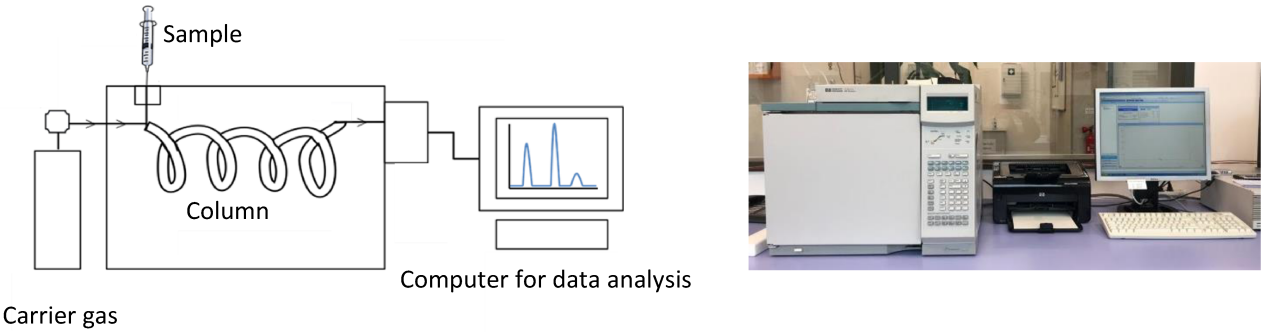


Figure 11 *Schematic representation and picture of Gas Cromatography apparatus (Torres, 2016)*

2.4. Calculation methods

All the equations reported in this chapter are from the VDI 4630 (Verein Deutscher Ingenieure e.V., 2016) and were used to quantify all the experimental results reported in this work.

2.4.1. Chemical analysis

As mentioned in chapter *2.3.1 TS, VS, PH, FOS/TAC*, the first analytical steps involve the calculation of the TS and VS. The formulas applied are:

$$TS = \frac{m_{105} - m_e}{m_t - m_e} * 100 \qquad (3)$$

TS: total solids [%]

m_{105}: mass of crucible + mass of sample after drying at 105°C [g]

m_e: mass of empty crucible [g]

m_t: mass of crucible + mass of sample before drying [g]

$$VS = \frac{m_{105} - m_{550}}{m_{105} - m_e} * 100$$

(4)

VS: volatile solids [%]

m_{105}: mass of crucible + mass of sample after drying at 105°C [g]

m_e: mass of empty crucible [g]

m_{550}: mass of crucible + mass of sample after ignition at 550°C [g]

2.4.2. Normal biogas production

The equation presented in this paragraph were used to quantify the volume of biogas in standard conditions, in each interval of time, specific to grams of volatile solids and the quantity related just to the degradation of the substrate. Through these calculations it was possible to elaborate the data collected from the daily biogas volume readings. As the pressure varies day by day in the climate room, the first step was to adjust the measured volume of biogas to normal condition of pressure and temperature. Hence, to calculate the normal volume of biogas the following formula was applied.

$$V_{tr,N} = V * \frac{(p - p_w) * T_N}{P_N * T}$$

(5)

$V_{tr,N}$: volume of the dry gas in the normal state [Nml]

V: volume of the gas as read off [ml]

P: pressure of the gas phase at the time of reading [hPa]

P_w: vapour pressure of the water as a function of the temperature of the ambient space [hPa]

T_N: normal temperature; TN = 273 K

p_N: normal pressure; p_N = 1013 hPa

T: temperature of the fermentation gas or of the ambient space [K]

The vapour pressure of water as function of temperature (Pw) was calculated through the Magnus Formula, assuming the biogas saturated with water vapour.

$$p_w = 6.11231 * e^{(17.5043*Tc/241.2+Tc)}$$

(6)

Tc: temperature of the gas in the climate room [°C]

*P*w: vapour pressure of the water as a function of the temperature of the ambient space [hPa]

For the GC analysis, 1 ml of biogas is necessary. So, when the biogas volume was measured after the GC analysis, 1 ml of biogas was added to the value read off.

2.4.3. Methane production

The equations presented in this chapter were applied to get the cumulative methane production and the production between intervals of measurements. The first step was to deduce the quantity of methane in the biogas from the quantity of methane resulted from GC analysis, that's necessary as the sample analysed from the headspace contain a mixture of nitrogen (coming from N_2 flushing), oxygen (possible presence of residues) and biogas. The formula used is the following one:

$$C_i = \frac{C'_i}{C'_{CH4} + C'_{Co2}} * 100$$

(7)

Ci: percentage of component (i) in biogas [vol %]

Ci': percentage of component (i) in headspace gas [vol %]

C'$_{CH4}$: percentage of methane in headspace [vol %]

C'$_{CO2}$: percentage of carbon dioxide in headspace [vol %]

Once the percentage of methane in biogas is calculate, the millilitres of methane produced easily deducible through the formula

$$V_{CH4} = V_{tr,N} * \frac{C_{CH4}}{100}$$

(8)

V_{CH4}: volume of produced methane [Nml]

$V_{tr,N}$: volume of the dry gas in the normal state [Nml]

C_{CH4}: percentage of methane in biogas [vol%]

The last step is to get the quantity of methane produced by the only consumption of substrate per one gram of volatile solids so that the results can be compared. It is important to consider both the volatile solids coming from the inoculum and from the substrate.

$$V_N = V_{CH4,R} - V_{specific\ CH4,B}\frac{m_{In,R}}{m_{Cell,R}}$$

(9)

V_N: specific volume of methane produced by substrate consumption per gram of volatile solids [NmL/gVS cell]

$V_{CH4,R}$: total volume of methane produced from reference samples [NmL]

$V_{specific\ CH4,B}$: volume of methane produced per gram of volatile solids coming from inoculum from blank samples [NmL/gVS IN]

$m_{In,R}$: mass of volatile solids coming from inoculum in reference samples [gVS IN]

$m_{Cell,R}$: mass of volatile solids coming from cellulose in reference samples [gVS Cell]

As mentioned in chapter *2.3.3 Biogas composition*, it was not possible to get the biogas composition values daily. The necessary values were calculated through linear interpolation.

$$y^n = \frac{CH_4^f - CH_4^i}{t^f - t^i} * \left(t^n - t^i\right) + CH_4^i$$

(10)

y_n: percentage of methane at time n [%]

CH_4^f: percentage of methane at the second measurement [%]

CH_4^i: percentage of methane at the first measurement [%]

t_f: time of the second measurement

t_i: time of the first measurement

t_n: time of the calculated measurement

To have a progressive and precise value of time, considering the hour and the day of the measurement, the formula applied is:

$$Time = (Day^n + Hour^n) - \left(Day^i + Hour^i\right)$$

(11)

Time: progressive value of time [days]

Day^n*:* day of the current measurement

Day^i*:* day of the first measurement

$Hour^n$*:* hour of the current measurement

$Hour^i$*:* hour of the first measurement

3. Experimental methods

In this chapter, the pre-tests carried out are described with the respective results. The first experiment considers four preservation strategies and gives a qualitative overview of their performances. The second, consists in a BMP test applied to compare the influence of MBRs material on the biogas production.

3.1. Pre-test on preservation strategies

The test reported in this chapter was carried out at the molecular biology laboratory of the Università degli Studi di Padova, under the supervision of Prof. Laura Treu. For this reason, most of the materials and analytical methods are different than the ones applied in the rest of the work.

3.1.1. Experimental set up

The preservation strategies considered are the following: freezing at 0 and -20°C with addition of DMSO as cryoprotectant, freezing at 0 and -20°C with addition of glycerol, agar gel as carrier at 0 and 20°C, gel rite as carrier at 0 and 20°C. Additionally, for each condition the performances using centrifuged inoculum and inoculum in its liquid medium were detected. To estimate the efficiency of preservation a Live/dead cell staining was performed on the samples and the biogas production after the revival was measured.
The used inoculum had the characteristics reported in table 8:

Table 8 *Characteristics of inoculum used for pre-test on preservation strategies.*

	TS [% WM]	**VS [% DM]**
Inoculum	2.756 ± 0.014	63.575 ± 0.241

Prior to preservation, it was cultivated for 7 days with cellulose (Sigma-Aldrich, CAS No.:9004-32-4) and starch (Sigma-Aldrich, CAS No.:9005-84-9) as substrate in BA medium prepared according to Angelidaki et al., 2009 and incubated at 37°C. Sodium sulfide, yeast extract and vitamins were added in a proportion of 1% volume. When the methane production was close to the maximal theoretical yield the inoculum was harvested.
In this experiment, cellulose and starch powder were not directly poured inside the inoculum bottles. Indeed, they were previously dissolved in BA medium. The considered proportion are: 2 g of cellulose

per 1 litre of BA medium, and 4 g of starch per 1 litre of BAm. Then, the two solutions were mixed, and the final solution was added to each inoculum bottle respecting the proportion 1g of cellulose and starch solution per 1gTS of inoculum.

A stock solution containing 50% volume glycerol (Carlo Erba reagents, Code n°453752) was prepared, flushed with nitrogen, sterilized by autoclaving for 20 minutes at 120°C and stored at ambient temperature.

For the DMSO a 100% stock solution was used and kept at ambient temperature.

As last, the two gels were prepared. For Agar (Agar-Millipore, CAS No.: 9002-18-0), 12 g of agar powder were dissolved in 1L of BA medium. For Gelrite (Sigma-Aldrich, CAS No.: 71010-52-1), 8 g of Gelrite powder in 1L of BA medium. After this, the solutions were flushed and autoclaved. Considering that these two compounds have gelatine texture at ambient temperature, it was necessary to wait until they cool down to around 50°C and use it before their solidification.

The experiment set up took place under an anaerobiosis hood represented in **Figure 14**. For the preparation of a centrifuged sample (P), 2 ml of inoculum were poured in an Eppendorf, centrifuged for 3 minutes at 3500 rmp and the liquid phase was removed. The solid phase was resuspended with 0.5 ml of BA medium and injected in anaerobiosis bottle. For not pelletized (NP) sample, the original cultivation media was kept, so the inoculum was directly injected in the anaerobiosis bottles.

Afterward, the stock solution was added based on the values reported in the table 9:

Table 9 *Specifics about quantities of stock solutions utilized in the pre-test on preservation strategies.*

Preservation strategy	Concentration of stock solutions	Concentration in stored samples	Correspondent volume added [mL]	
			P	NP
Glycerol	50% v/v	25% v/v	0.5	0.8
DMSO	100% v/v	5% v/v	0.025	0.04
Agar gel	12 g/L	6 g/L	0.5	0.8
Gelrite	8 g/L	4 g/L	0.5	0.8

Finally, the samples were stored for 20 days. The ones preserved in Agar and Gelrite were stored at 0 and 20°C, the preserved in DMSO and glycerol were kept at 0 and -20°C.

Table 10 Experimental setup of the pre-test on preservation strategies.

Preservation medium	Pelletization	Temperature
0.8 ml inoculum+ 0.8 ml Glycerol solution	NP	0 and -20°C
0.8 ml inoculum+ 0.04 ml DMSO	NP	0 and -20°C
0.8 ml inoculum+ 0.8 ml Agar gel solution	NP	0 and 20°C
0.8 ml inoculum+ 0.8 ml Gelrite solution	NP	0 and 20°C
0.5 ml inoculum+ 0.5 ml Glycerol solution	P	0 and -20°C
0.5 ml inoculum+ 0.025 ml DMSO	P	0 and -20°C
0.5 ml inoculum+ 0.5 ml Agar gel solution	P	0 and 20°C
0.5 ml inoculum+ 0.5 ml Gelrite solution	P	0 and 20°C

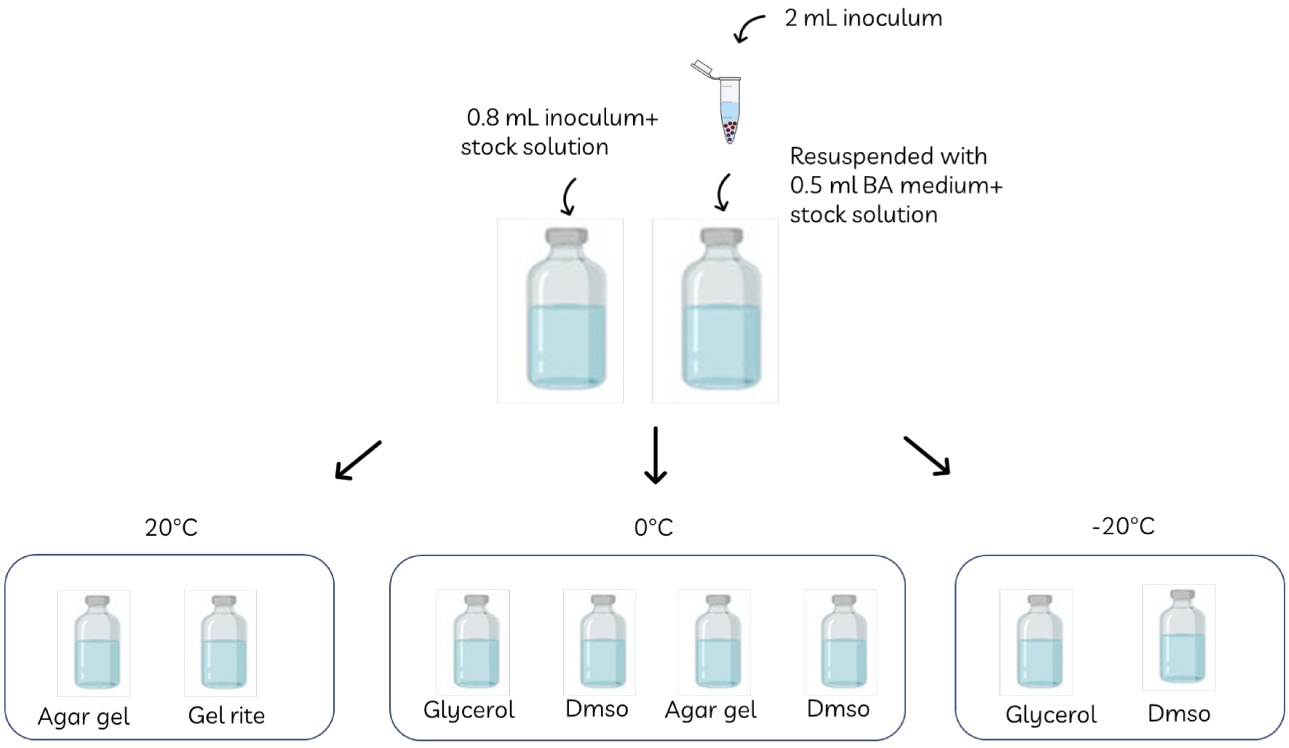


Figure 12 Flowchart of the methodology applied for the pre-test on preservation strategies.

The main tools and machineries used for the experimental set up are reported in the following images.

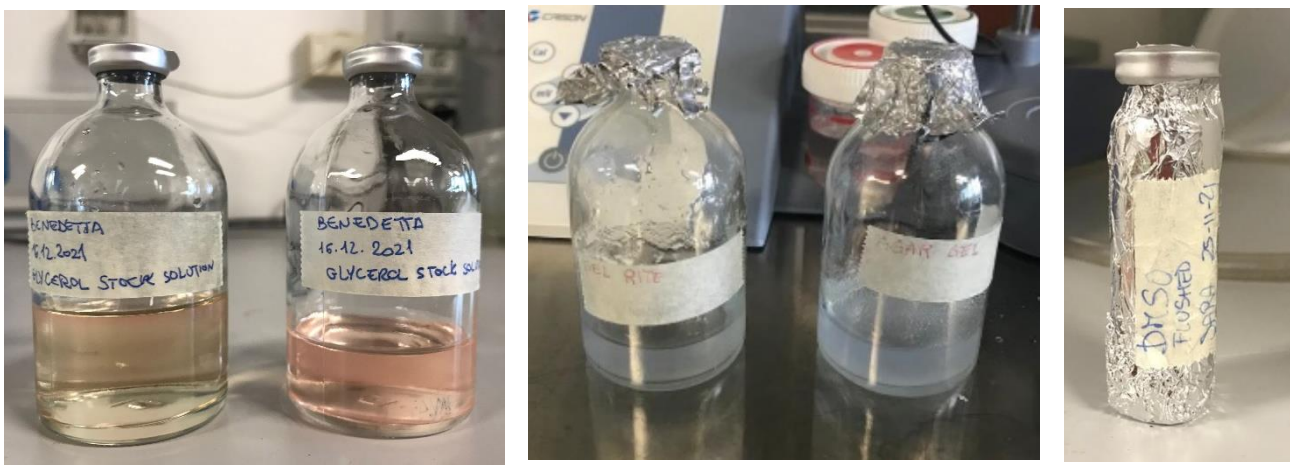


Figure 13 *Pictures representing respectively: glycerol stock solution, gelrite and agar gel solutions, DMSO solution.*

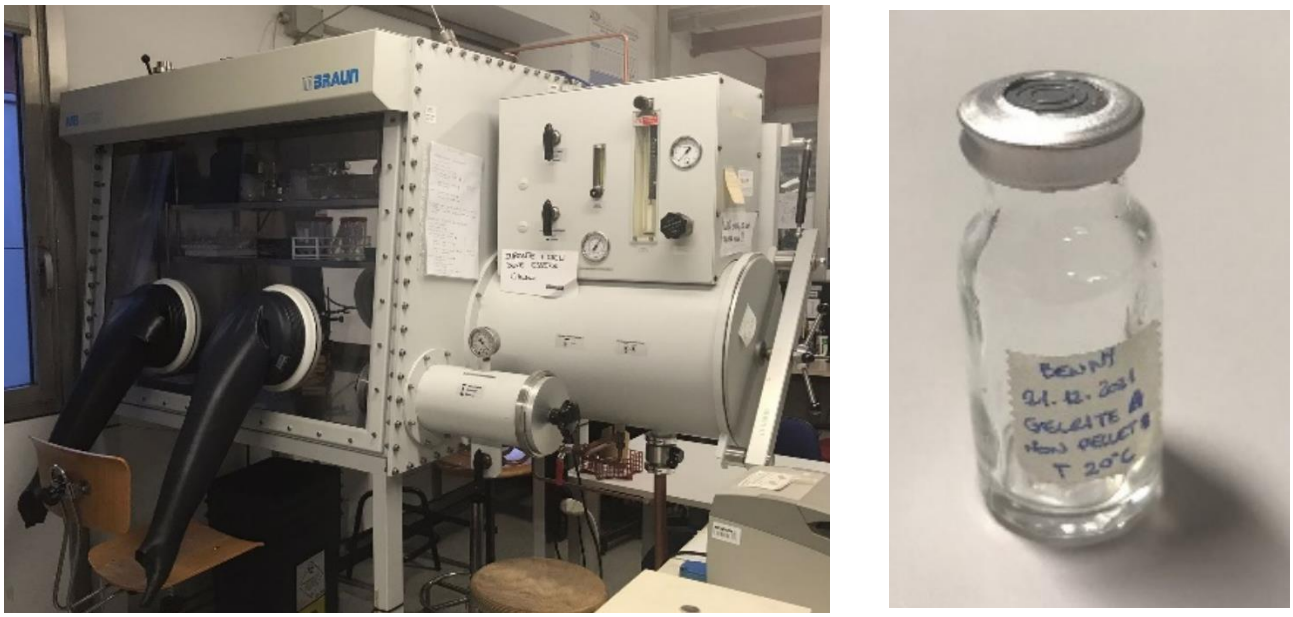


Figure 14 *Pictures representing respectively the anaerobiosis hood and the bottles used as container for the stored samples.*

3.1.2. Live/Dead cell staining

Before and after the storage, the efficiency of preservation was evaluated using Fluorescein Diacetate-Propidium Iodide double staining. Spectrophotometric determination of the hydrolysis of fluorescein diacetate (FDA) was shown to be a simple, sensitive, and rapid method for determining microbial activity in soil and litter (Schnurer & Rosswall, 1982). FDA is hydrolysed by several enzymes, such as proteases, lipases, and esterases. The product of this enzymatic conversion is fluorescein, which can be visualized inside cells by fluorescence microscopy. Propidium iodide (PI) is widely used for bacterial viability staining, as it can cross compromised bacterial membranes and stain DNA and RNA inside of dead cells or the ones with reversibly damaged membranes (Rosenberg et al., 2019). Therefore, in viable cells, FDA can be converted into fluorescein by esterase. Propidium Iodide (PI) can interact with the DNA after penetrating dead cells. FDA-PI double staining can simultaneously evaluate viable cells and dead cells. As a result, through fluorescence microscopy alive cells are clearly visible in green and dead are highlighted in red.

FDA stock solution was prepared dissolving 5 mg of FDA in 1 ml of acetone. For PI stock solution, 1 mg of PI was added to 1 ml of milliQ water. Finally, the FDA-PI solution was prepared mixing 20µl of FDA stock and 50 µl of PI stock to 9.93 mL of Phosphate-buffered saline (PBS, Fluka BioChemika, 79382). All the solutions must be stored at 4°C and be prepared by keeping the containers of the different stocks on ice and not exposed to light to avoid photosensitization.

Immediately before the microscope analysis, to 10 µl of inoculum was added 990 µl of milliQ water (proportion 1:100 volume) and centrifuged. After the removal of water, 100 µl PBS was added to the remaining solid phase and centrifuged again. Lastly, 100 µl of FDA-PI solution was mixed with the pelletized sample and kept in the dark for 5 minutes. After that the culture was ready for the fluorescence microscope analysis. The microscope used is reported in **Figure 15** (Leica Microsystems DM6 FS).

Figure 15 *Fluorescence microscope used for Live/dead cell staining at the biology laboratory.*

3.1.3. Results and discussion

The results of the analysis on the culture before storage are reported in **Figure 16**. Most cells were alive as confirmed by the high biogas production

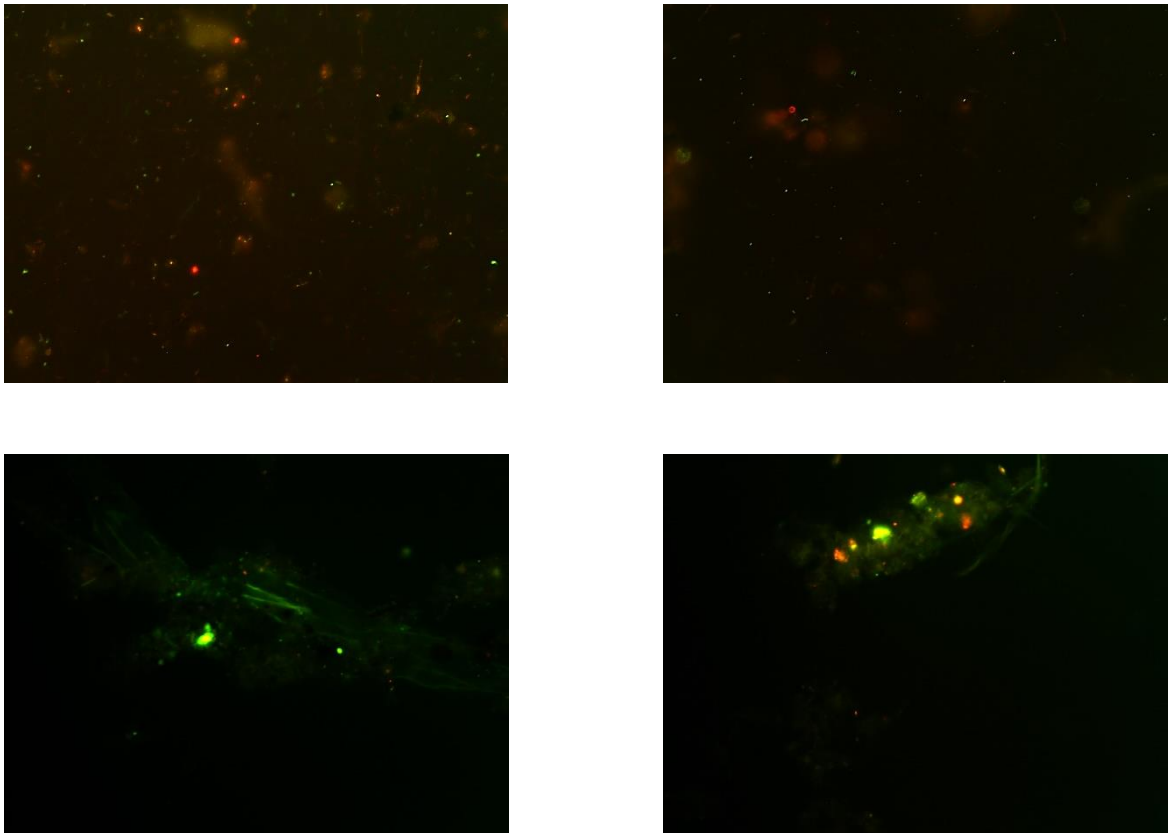


Figure 16 *Live/dead cell staining pictures representing the spatial distribution of microorganisms before storage, during the pre-test on preservation strategies.*

Immediately after the 20 days preservation, the pictures in table 11 were obtained from Live/dead cell staining. From a qualitative evaluation, it is not possible to state if pelletization had a negative effect on the inoculum. Therefore, even if on one side it increases the biomass concentration, it could cause stress induced by the centrifuge and the interaction with oxygen. Considering the two cryoprotectants, the preservation through Glycerol was more efficient than the one with DMSO. This first assumption was confirmed by the results coming from the biogas production, indeed all the samples treated with DMSO did not produce biogas. For both the strategies in the samples at -20°C more clusters of alive cells were observed.

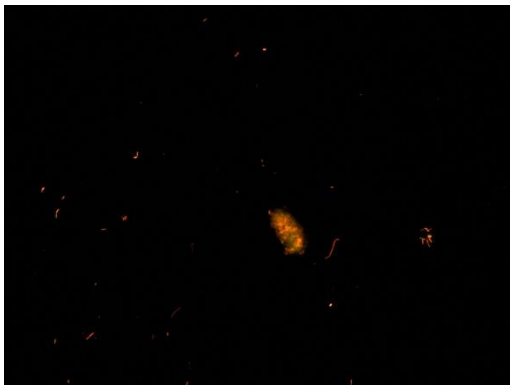
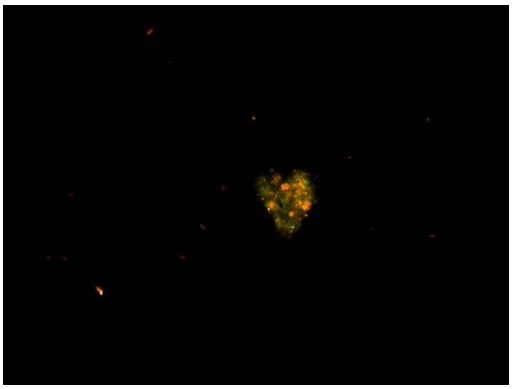
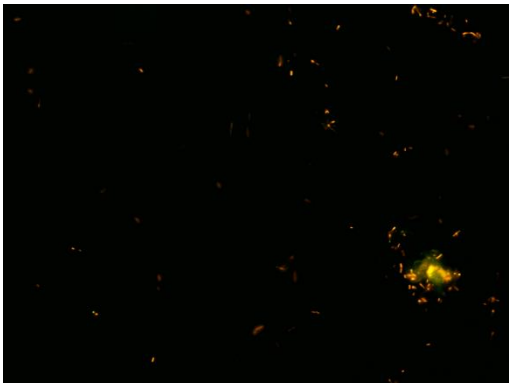
The temperature of 0°C was proven to be not suitable for preservation in gels, probably because of the damage caused by partial freezing. There was no visible difference in the preservation effectiveness between the two gels.

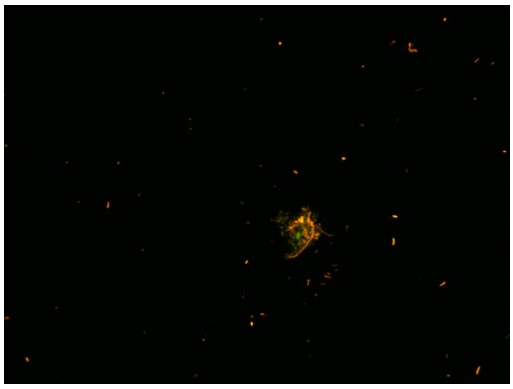
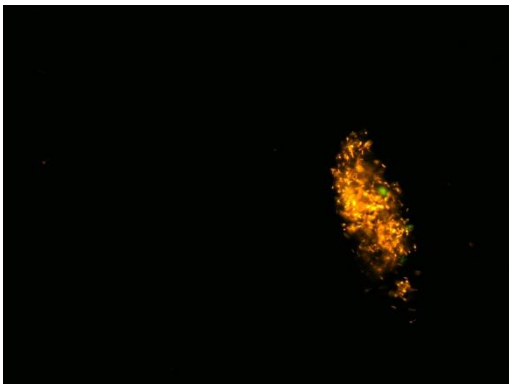
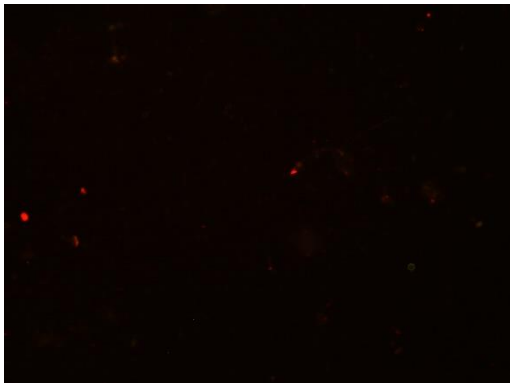
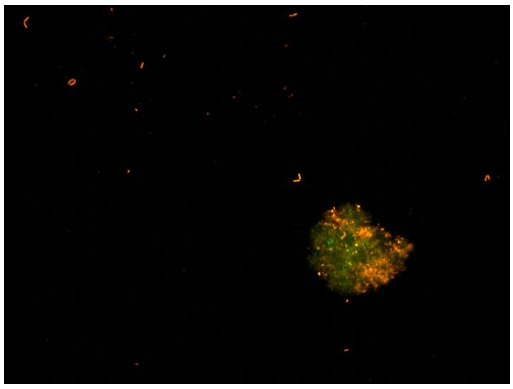
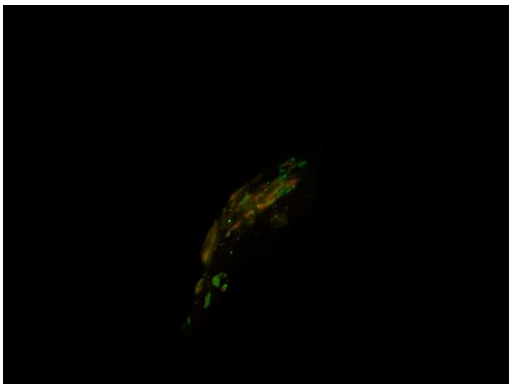
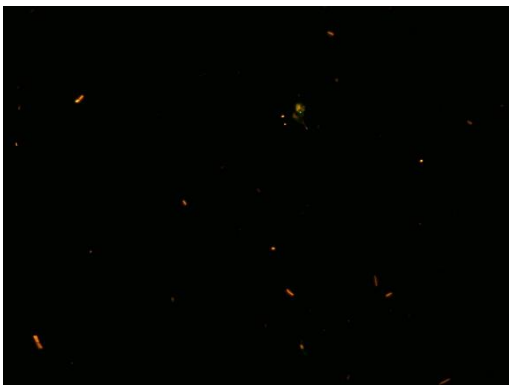
For the revival, in each sample cellulose and starch were added and the bottles were incubated at 37°C on a continuous stirrer. It took two weeks to get a detectable quantity of biogas. The produced volume could not be precisely measured during all the reactivation process, so the obtained values are considered just as a qualitative indication of the preservation efficiency. The strategy that leaded to the best revival was glycerol, both for samples pelletized and not pelletized, with good results for 0 and -20°C. Agar gel and gelrite showed good performances, but with a biogas production lower than glycerol.

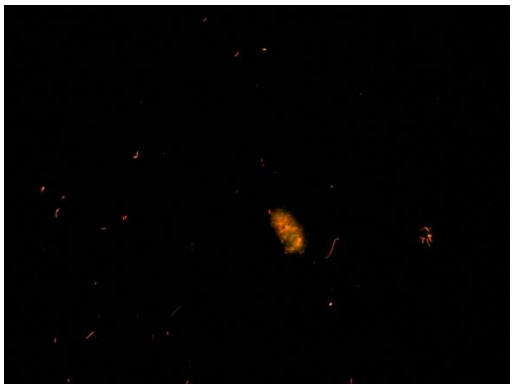
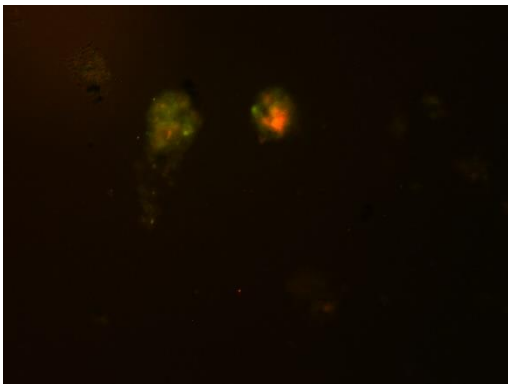
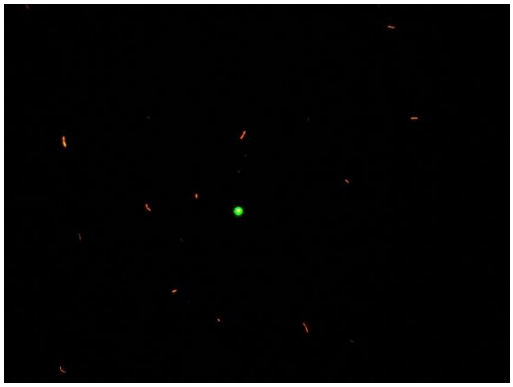
From a general point of view, samples stored with glycerol and gels resulted in a higher number of viable cells. In samples stored with DMSO, some clusters of alive cells were found, but no production of biogas was detected.

The further work was focused on the preservation performance of agar gel at 20°C and DMSO at -20°C. Considering the results of this qualitative analysis, in the new experiment more inoculum was used for the preservation.

Table 11 *Live/dead cell staining pictures representing the spatial distribution of microorganisms after storage, during the pre-test on preservation strategies.*

		P	NP
GLYCEROL	-20°C		
	0°C		

DMSO	-20°C		
	0°C		
AGAR GEL	20°C		
	0°C		

GELRITE	20°C		
	0°C		

3.2. Pre-test on MBRs materials

Another analysis was carried out prior the final test. The aim was to evaluate the performance of MBRs made of glass, Teflon (FEP), and Polypropylen Copolymer (PPCO). The experiment was set up considering 3 blanks and 3 reference replicates for each type of MBR. This resulted in 18 reactors, of which 6 in glass, 6 in Teflon and 6 in PPCO.

The first step was checking the tightness of the reactors as described in chapter *2.2.1 Minibioreactors*. Once it was verified that all the reactors were working properly, in each one 12 g of previously sieved sludge was poured. For the reference samples, 0.065 g of cellulose as substrate were added. The quantity of substrate to add was calculated considering a ISR between 2 and 4. From Total solids and volatile solids analysis, for this sample of digestate and cellulose we got the following values:

Table 12 *Characteristics of inoculum used for the pre-test on MBRs materials.*

	TS [% WM]	**VS [% DM]**
Sieved inoculum	3.049 ± 0.018	64.456 ± 0.435
Cellulose	95.743 ± 0.107	99.936 ± 0.039

Therefore, the following calculation was considered:

$$ISR = \frac{VS\ inoculum}{VS\ substrate} = \frac{12 * 3.049\ * 64.456}{0.065 * 95.743 * 99.936} = 3.7922$$

First, the bottle containing the inoculum to be used was put on a stirrer to ensure homogeneity between all the replicates. So, the established quantity of inoculum was withdrawn from the bottle with the help of a syring and injected inside each MBR. Cellulose was added to the references. Then, the headspace was purged with nitrogen for one minute to create anaerobic conditions. To flush with nitrogen, the MBR cap was closed loosely, and the rubber septum was pierced with a needle connected to a nitrogen sink. After that, the samples were closed tightly and shacked on a minishaker, and finally incubated at 37±1°C. After an hour, the excess of pressure was released, and the experiment started running. Releasing the gas before the beginning of the test, make it possible to avoid further overpressure caused by flushing with nitrogen and expansion of the gases at 37°C. In some cases, gas expansion due to change in temperature is not completely over after an hour, and this bring to overestimation of the biogas production in the next measurement. Two pictures representing the MBRs prior to incubation is reported in **Figure 17**.

Figure 17 *Pictures representing the three different types of MBRs. In the first position are glass, in second FEP, in third PPCO.*

The experiment run for 24 days during which biogas volume was measured daily and GC analysis was carried out two-three times per week. The test was shut down when daily biogas production during three consecutive days was less than 1% of the accumulated volume of biogas. It is important to point out that at each volume measurement current day, time, temperature, and pressure were stated.

From a first sight to the volume of methane produced per gram VS (**Figure 18**), just the results coming from samples in glass MBR can be accepted as they are the only one in which the BMP is higher than 340 $NmLCH_4/gVS$ (as stated in Holliger et al., 2021). However, the cause of this difference lies in the FEP and PPCO permeability to CO_2. As visible in **Figure 19**, **Figure 20**, and **Figure 21**, all the curves representing CH_4 and CO_2 concentration in percentage, are similar except for the one representing CO_2 in references. Only this curve has decreasing trend after the 6^{th} day for glass and PPCO MBRs. Specifically, in glass and PPCO MBRs when the carbon dioxide reaches around 35% of the biogas volume, its quantity starts decreasing over time. The potential explanation proposed is that when the CO_2 concentration in the headspace biogas reach 35% of the volume, the carbon dioxide escape through the plastic MBRs walls due to the increased pressure and the small dimension of this molecule. The permeability of N_2, O_2 and CO_2 through FEP and PPCO are reported in table 13. Carbon dioxide is the gas with the highest permeability for both the materials, so it has the highest rate of transportation through the plastic MBRs walls per unit of time, surface, and pressure.
Considering the results of this pre-test, it is preferable to use glass MBRs whenever it is possible. However, also FEP and PPCO reactors were utilized in the final test because of the lack of material.

Table 13 *Permeability of nitrogen, oxygen, and carbon dioxide through FEP and PPCO.*

	FEP [cc.-mm/ m2-24 hr.-Bar]	PPCO [cc.-mm/ m2-24 hr.-Bar]
N_2	124.34	17.48
O_2	291.41	77.71
CO_2	845.42	252.56

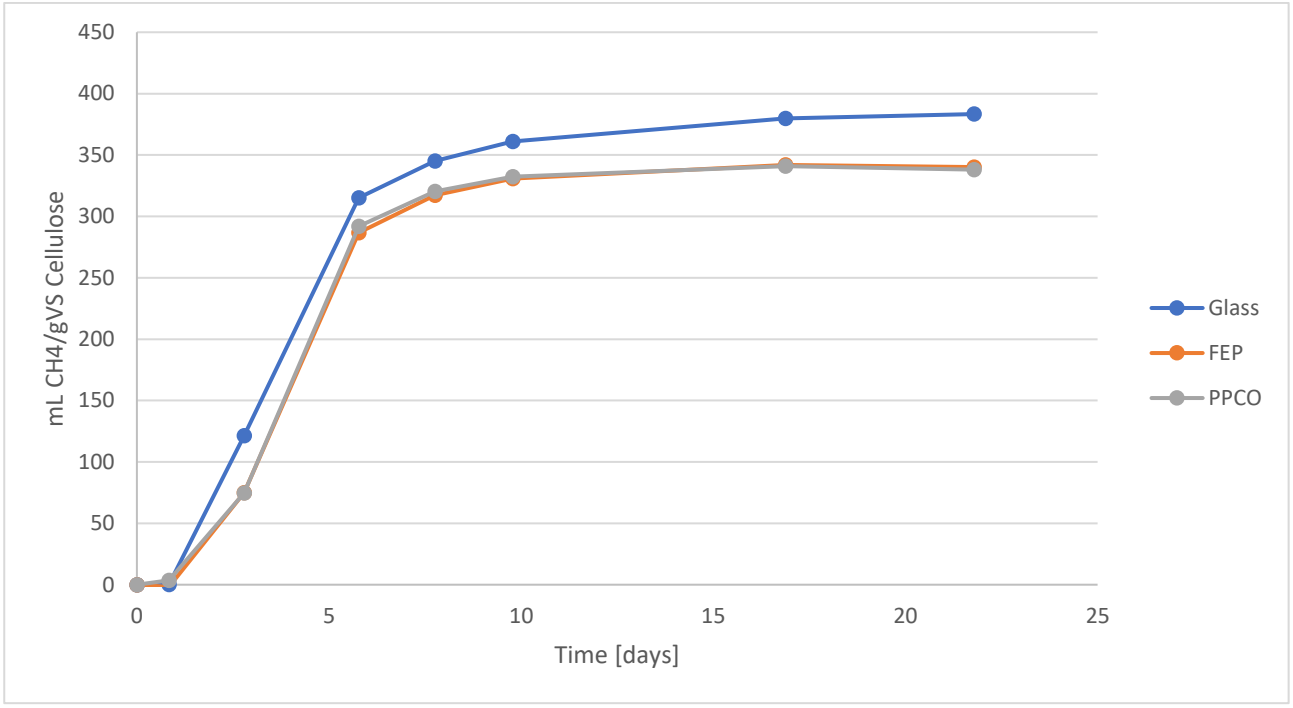


Figure 18 *Cumulative volume of methane produced due to consumption of cellulose per gram of volatile solid during the MBRs materials pre-test.*

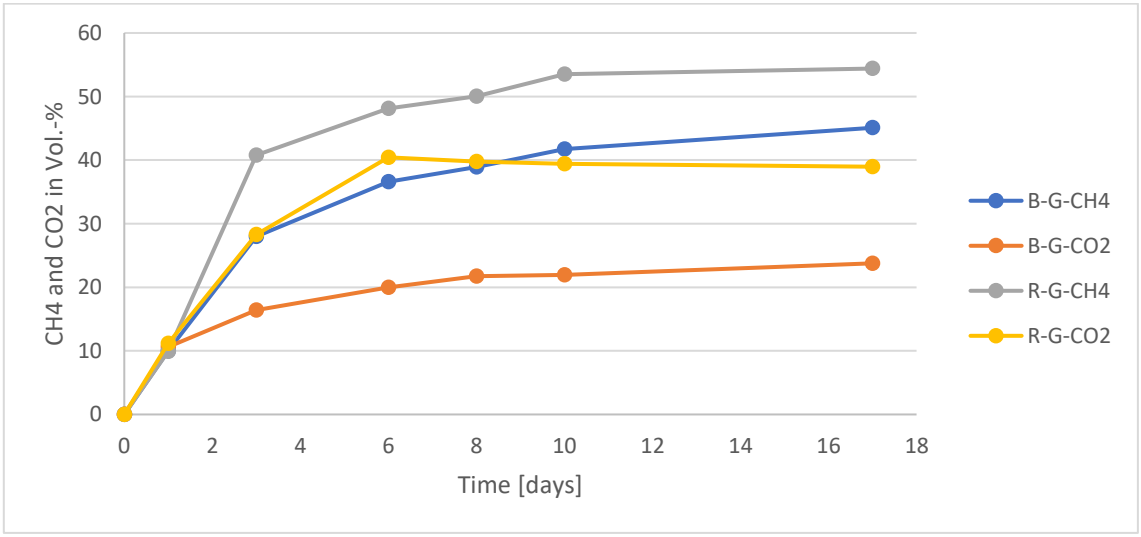


Figure 19 *Biogas composition changes over time in the glass MBRs.*

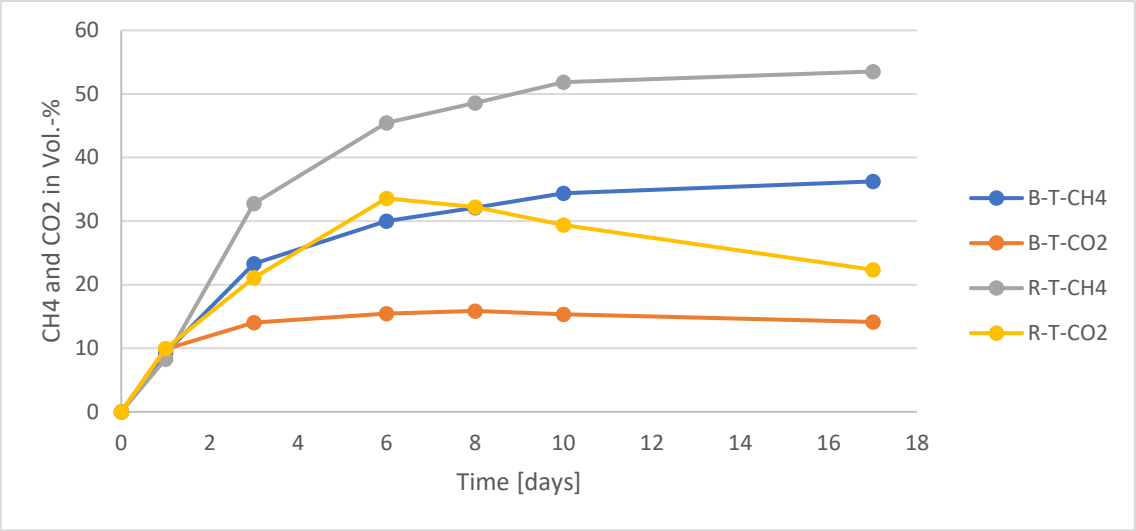


Figure 20 *Biogas composition changes over time in the FEP MBRs.*

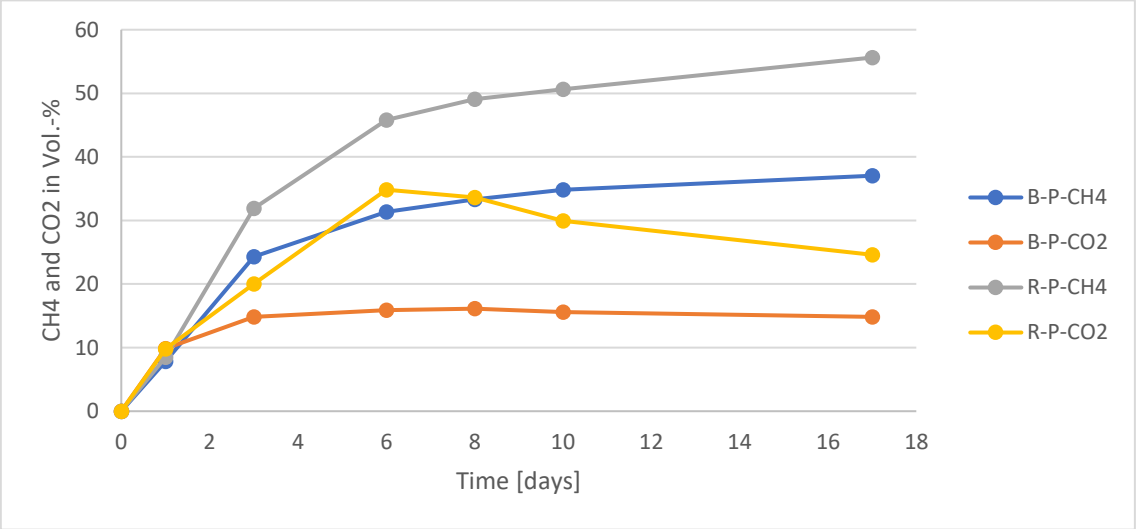


Figure 21 *Biogas composition changes over time in the PPCO MBRs.*

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SECOND PART

Scientific paper

SECOND PART

Abstract

Accurate biochemical methane potential (BMP) tests are essential to find new, efficient substrates and optimize existing processes to get higher quantity and better-quality biogas. Even if consistent protocols exist, the major bottleneck in this research is the lack of a ready-to-use, standardized methanogenic culture inoculum. The cause is the slow growth of microorganisms in anaerobic conditions and the high cost of maintaining them active in the necessary amount. This study considers two preservation strategies: freezing at -20°C with addition of DMSO as cryoprotectant and the use of agar gel as preservation carrier stored at 20°C. The results of the BMP test after storage indicates that agar gel represents the most efficient preservation strategy, but also reveals that agar is consumed by microorganisms as substrates even during the storage period. DMSO has a detrimental effect on the samples as it turns out to have high toxicity at incubation temperature.
To investigate the effect of DMSO, a further experiment was set up. The samples were stored for one night at -20°C degrees with the addition of DMSO. The following day in half of the samples it was removed by centrifugation, in the other half it was left. Finally, a BMP test was performed and showed that removal of DMSO through centrifugation enhances the revival, but the inoculum doesn't reach its usual methane production.

1. Introduction

Not only climate change, but also the strong European dependence on undergrown stocks of gas and rising energy prices show that an alternative to fossil energy imports is needed. The direct replacement of gas by renewables is represented by biogas from anaerobic digestion, as this process converts organic waste into easily disposable material with production of energy. However, even if the production pathway has been widely studied, the applied technology is still not advanced enough to supply energy to a large population and be competitive. In this frame, Biochemical methane potential (BMP) tests are important to find new, efficient substrates and optimize the process. Standardization of BMP is necessary to ensure inter laboratory repeatability and accuracy since the results are used also to assess the monetary value of substrate and performance of anaerobic digesters. Guidelines for standardized procedures exist (Angelidaki et al., 2009; Holliger et al., 2021; Verein Deutscher Ingenieure e.V., 2016). Fixed methods are proposed as concerns substrate and inoculum characterization, experimental procedure and data collection, interpretation, and reporting. However,

no recommendations about the methanogenic community composition or abundance are established. Therefore, a certain degree of variability in the results always remains due to different microbial populations that leads to different activities, quantities of endogenous methane production and substrate adaptation. In this context, it is crucial to find an efficient technique to store bacterial seeds for long times and guarantee easy transportation of the samples. It is also necessary to ensure the quick revival and the same performance of inoculum after preservation.

Based on previous developments (Heerenklage et al., 2017), this study compares the performance of BMP tests on samples after storage for 14 days at -20°C with the addition of DMSO as cryoprotectant and samples stored at 20°C with the addition of agar gel as carrier.

The efficiency of the preservation was evaluated considering the specific methane produced by the degradation of cellulose (used as substrate for the inoculum in the revival test) and comparing biomethane produced from samples stored with agar gel/DMSO and samples stored at the same temperature without any carrier or cryoprotectant.

As all the samples treated with DMSO showed a great reduction of biogas production and long lag-phase, a further investigation was carried out to examine the toxicity of DMSO at 37±1°C (the temperature at which the revival takes place).

2. Material and methods

2.1. Digestate inoculum and substrate

The inoculum was collected from the mesophilic anaerobic digester of a municipal wastewater treatment plant (Klärwerk Hamburg-Köhlbrandhöft). After sieving through 1 mm mesh screen, total solids (TS), volatile solids (VS), pH and FOS/TAC were detected according to the standard DIN 15935 (Deutsches Institut für Normung e.V., 2012). Since in the BMP test cellulose was used as substrate, it was also analysed for TS and VS. The results are reported in table 14.

Before the experimental set up, the digestate was incubated for two weeks at 37±1 °C to enhance the degradation of residual organic material.

Table 14 The characteristics of inoculum and substrate.

Parameter	Inoculum	Substrate
TS [% WM]	1.912 ± 0.003	95.743 ± 0.107
VS [% DM]	63.546 ± 0.049	99.936 ± 0.039
PH	7.8	-
FOS/TAC	0.197	-

The ideal quantity of cellulose to add in the references, was assessed considering the Inoculum to substrate ratio (ISR) defined according to Holliger et al., 2016. To avoid acidification or inhibition problems ISR should be always greater than 1, and for most of the applications between 2 and 4. in our case the following calculation was considered:

$$ISR = \frac{VS\ inoculum}{VS\ substrate} = \frac{12 * 1.912\ * 63.546}{0.051 * 95.743 * 99.936} = 2.989$$

Thus, for 12 gTS of fresh inoculum, 0.051 gTS of cellulose were added.

2.2 Experimental set-up

2.2.1 Preservation strategy

The tests were carried out following mainly two studies (Yan et al., 2020; Yan et al., 2021).
Two different preservation strategies were investigated: storage of inoculum in agar solution at 20°C (AGAR 20°C) and storage with DMSO at -20°C (DMSO -20°C). To understand the influence of DMSO and agar gel, a certain quantity of inoculum was stored as well at 20 and -20°C without addition of cryoprotectant or gel (ZERO 20, ZERO -20).
Glass Minibioreactors (MBRs) with 34 ml volume were used for all the samples, except for the DMSO assay; in this case plastic MBRs with 43 and 46 ml volumes were used. Prior to preservation it was necessary to check the tightness of the system. Therefore, once the apparatus was sealed, 10 ml of water and 5 ml of atmospheric air were injected in each reactor. The following day the inner gas volume was measured and compared with the injected volume, the MBRs in which these two volumes were found to be different were discarded. The last step before the experiment set up, was to purge nitrogen in each reactor, to remove as much oxygen as possible.

For strategy AGAR 20°C, the inoculum was centrifuged for 15 minutes at 4000 rpm (3K18 centrifuge from SIGMA) and the liquid phase was removed. The agar solution (concentration of agar powder 10 g/L) was prepared heating 150 ml of basal anaerobic medium (BA medium) (Angelidaki et al., 2009) to 90°C; 1.5 g of agar powder (VWR International, CAS No.: 9002-18-0) were poured inside the medium and continuously stirred. The solution was maintained at 90°C until the agar powder was completely dissolved, then it was cooled down to 50°C and added in each MBR. The ratio 1 gTS of centrifuged inoculum per 1 g of agar solution was reached.

For strategy DMSO, the inoculum was pelletized as previously described. The liquid phase was removed and BA medium plus DMSO (VWR International, CAS No.: 67-68-5) were added. As suggested in literature (Hoefman et al., 2012) (Yu & Quinn, 1994), the addition of DMSO was 5% of the final volume, so the concentration reached was 0.05g/L.

To prepare ZERO 20°C and ZERO -20°C samples, the inoculum was centrifuged and after the removal of liquid phase, BA medium was added.

All the specifics related to the mass input are reported in table 15 and the preparation is summarized in **Figure 22**.

For each of the conditions, six replicates were prepared and stored. After the preparation, the reactors were flushed with nitrogen for 1 minute and stirred. Finally, they were stored for 14 days at the established temperatures.

Contextually to the beginning of preservation period, the positive control was set up to investigate the influence of DMSO and agar gel on the microbial community at 37±1°C and to check the activity of inoculum. The samples were prepared in the same conditions as those stored, for each condition four replicates were prepared and later divided into references (with addition of cellulose as substrate) and blanks (without substrate) and incubated without any storage period.

2.2.2 Revival test

After 14 days at the established temperatures, the second BMP test was performed on the preserved samples. Specifically, the six replicates were divided in three samples representing the references and three representing the blanks. After the addition of cellulose to the references, the samples AGAR 20°C and ZERO -20°C were flushed with nitrogen, mixed, and incubated at 37 ±1 °C on a continuous shaker.

The process was different for samples containing DMSO as from the positive control and literature studies (Hoefman et al., 2012), it was deduced its strong toxicity at 37°C. Therefore, the samples were thawed at ambient temperature for 10 minutes, then liquid phase was removed by centrifugation for 15 minutes at 4000 rmp and replaced by the same quantity of BA medium.

Table 15 *The characteristics of each experiment group.*

Strategy	Preservation medium	Temperature	Revival
AGAR	3.5 g pelletized inoculum 3.5 g agar solution	20°C	0.051 g cellulose in references
DMSO	3.5 g pelletized inoculum 3.5 g BA medium 0.35 g DMSO	-20°C	Liquid phase replaced with 3.85 g BA medium 0.051 g cellulose in references
ZERO	3.5 g pelletized inoculum 3.5 g BA medium	20 and -20°C	0.051 g cellulose in references

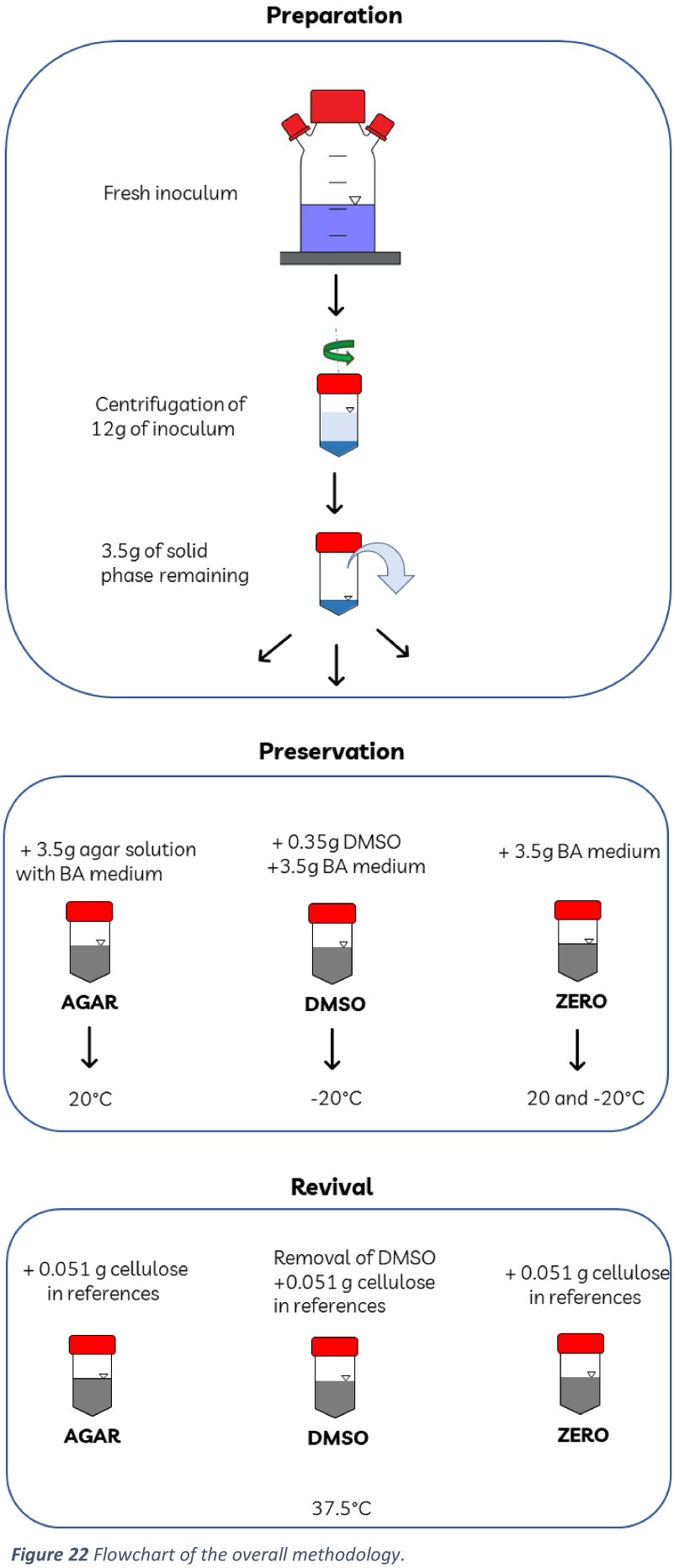


Figure 22 *Flowchart of the overall methodology.*

2.2.3 DMSO toxicity assay

Since from positive control DMSO resulted toxic, an analysis of its influence was conducted, specifically the efficiency of DMSO removal by centrifugation was investigated. Samples with centrifuged sludge were prepared, adding BA medium and 5% DMSO. They were stored for one night at -20°C, then in half of them DMSO was removed by centrifugation and the liquid part replaced by BA medium, in the other half it was kept in the reactor. Blanks and reference samples were prepared adding cellulose as described and the BMP run in the same condition as described before.

Table.16 *Experimental set up of the DMSO toxicity assay.*

Strategy	Preservation medium	Temperature	Revival
YES DMSO	3.5 g pelletized inoculum 3.5 g BA medium 0.35 g DMSO	-20°C	0.051 g cellulose in references
NO DMSO	3.5 g pelletized inoculum 3.5 g BA medium 0.35 g DMSO	-20°C	Liquid phase replaced with 3.85 g BA medium 0.051 g cellulose in references

2.3 Analytical methods

2.3.1 Chemical analysis

PH, TS, VS and FOS/TAC were determined according to the standard DIN 15935 (Deutsches Institut für Normung e.V., 2012).

2.3.2 Biogas volume

The biogas produced by each sample was quantified using a volume reader apparatus (reported in **Figure 23**) composed of a needle connected to the top of a graduated glass pipette; the pipette is kept in a cylinder filled with a barrier solution (Polydimethyl-Siloxane, Fa. Ritter, Germany) and it has an opening on the bottom so that the liquid can enter the pipette. When needle pierces the rubber septum of the MBR, biogas accumulated in the reactor is driven into the pipette, where because of the increased pressure, the liquid level decrease and make it possible to read the detected volume of

biogas. All the formulas applied in this calculations come from VDI 4630 (Verein Deutscher Ingenieure e.V., 2016).

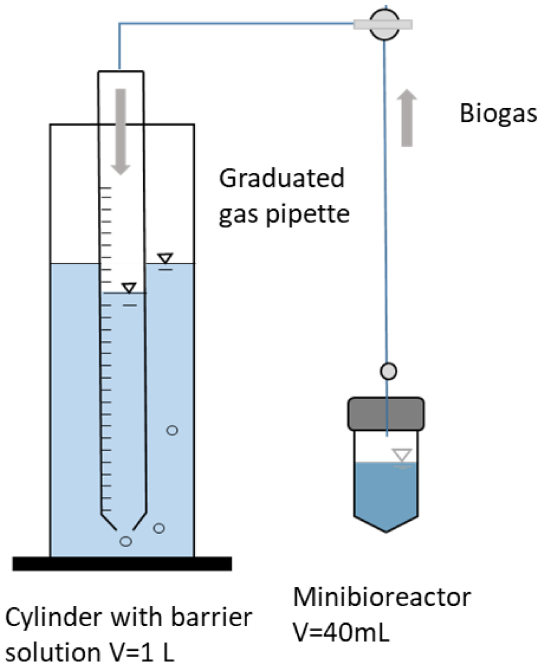


Figure 23 Schematic representation of the volume reader apparatus.

As the pressure may have significant variations day by day, the measured volume of biogas was adjusted to normal condition of pressure and temperature using the following formula.

$$V_{tr,N} = V * \frac{(p - p_w) * T_N}{P_N * T} \quad (1)$$

$V_{tr,N}$: volume of the dry gas in the normal state [Nml]

V: volume of the gas as read off [ml]

P: pressure of the gas phase at the time of reading [hPa]

P_w: vapour pressure of the water as a function of the temperature of the ambient space [hPa]

T_N: normal temperature; TN = 273 K

p_N: normal pressure; p_N = 1013 hPa

The vapour pressure of water as function of temperature (Pw) was calculated through the Magnus Formula, assuming the biogas saturated with water vapour.

$$p_w = 6.11231 * e^{(17.5043*Tc/241.2+Tc)}$$

(2)

Tc: temperature of the gas in the climate room [°C]

*P*w: vapour pressure of the water as a function of the temperature of the ambient space [hPa]

2.3.3 Methane production

The percentage of methane and carbon dioxide in the biogas were detected using a Gas Chromatograph, specifically the one used in this work is Agilent/HP 6890 GC. Thanks to this analytical technique it was possible to get the precise percentage of each chemical components in the headspace of the MBR.

The data were computed using the following formulas.

As the headspace gas contains a mixture of nitrogen (coming from N_2 flushing), oxygen (possible presence of residues) and biogas, to deduce the real percentage of methane in biogas the formula used is:

$$C_i = \frac{C'_i}{C'_{CH4} + C'_{Co2}} * 100$$

(3)

Ci: percentage of component (i) in biogas [vol %]

Ci': percentage of component (i) in headspace gas [vol %]

C'$_{CH4}$: percentage of methane in headspace [vol %]

C'$_{CO2}$: percentage of carbon dioxide in headspace [vol %]

Once the percentage of methane in biogas is calculate, the millilitres of methane produced are easily deducible through the formula

$$V_{CH4} = V_{tr,N} * \frac{C_{CH4}}{100}$$

(4)

V_{CH4}: volume of produced methane [Nml]

$V_{tr,N}$: volume of the dry gas in the normal state [Nml]

C_{CH4}: percentage of methane in biogas [vol%]

The last step is to get the quantity of methane produced by the only consumption of substrate per one gram of volatile solids, this result is obtained subtracting the volume of methane measured in

references minus the volume in blanks. It is important to consider both the volatile solids coming from the inoculum and from the substrate.

$$V_N = V_{CH4,R} - V_{specific\ CH4,B} \frac{m_{In,R}}{m_{Cell,R}} \quad (5)$$

V_N: specific volume of methane produced by substrate consumption per gram of volatile solids [NmL/gVS cellulose]

$V_{CH4,R}$: total volume of methane produced from reference samples [NmL]

$V_{specific\ CH4,B}$: volume of methane produced per gram of volatile solids coming from inoculum from blank samples [NmL/gVS Inoculum]

$m_{In,R}$: mass of volatile solids coming from inoculum in reference samples [gVS Inoculum]

$m_{Cell,R}$: mass of volatile solids coming from cellulose in reference samples [gVS Cellulose]

3. Results and discussion

3.1. Positive control

From the positive control, it is possible to deduce how the substances added for each strategy affects the biogas production and composition at incubation temperature. **Figure 24** shows the cumulative quantity of biogas produced during the positive control, it is clearly visible that both blanks and references samples treated with agar have a much higher production than others. The higher gas production in B-Agar than in B-Zero samples is caused by the utilization of agar gel as substrate by methanogenic cultures, as will be confirmed by further analysis. DMSO toxicity can be assumed, as the gas production is much lower than the zero samples both for blanks and references. These results are confirmed by the biogas composition at the last measurement (**Figure 25**). Anaerobic digestion is highly affected by DMSO presence and even at the end of the process the percentage of methane in headspace gas is both for blanks and reference 4%, while CO_2 is 25% for blanks and 48% for reference. **Figure 26** represents the net methane produced by the consumption of cellulose per grams of volatile solids and corroborates the mentioned hypotesys.

In conclusion, in absence of preservation the qunatity of methane produced by only the inoculum is 253.587 Nml CH_4/gVS. Considering samples treated with agar, we had that the consumption of 0.035gTS of agar powder caused an increasment of 100 NmlCH_4/gVS of the BMP. Samples contsining DMSO produced around 220 NmlCH_4/gVS less then the not treated inoculum.

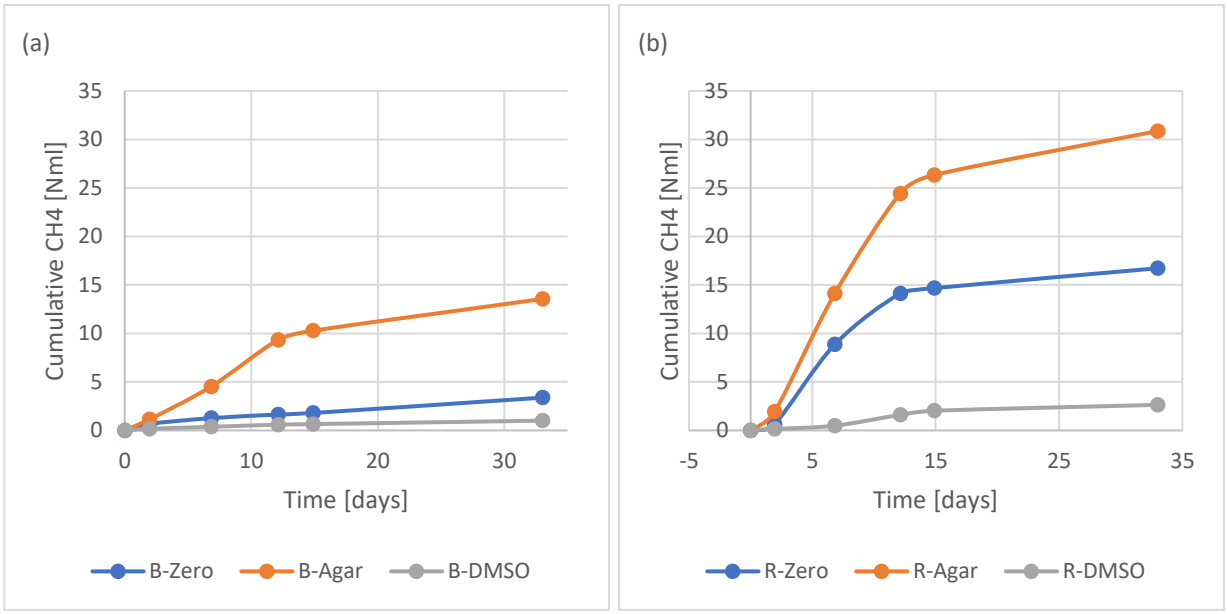


Figure 24 *Cumulative volume of methane produced during the positive control in blank (a) and reference (b) samples.*

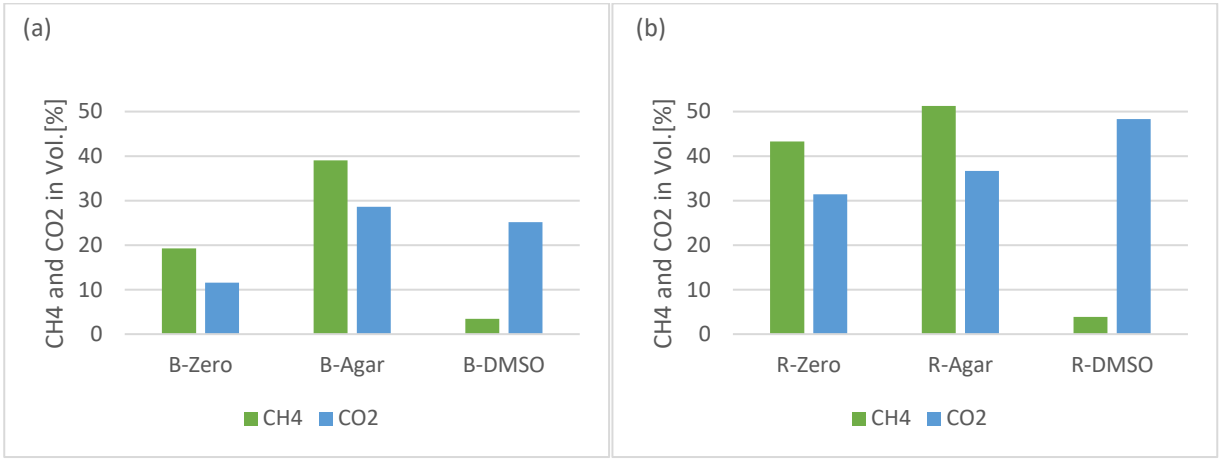


Figure 25 *Percentage of methane and carbon dioxide in biogas from MBR headspace measured at the end of the positive control in blank (a) and reference (b) samples.*

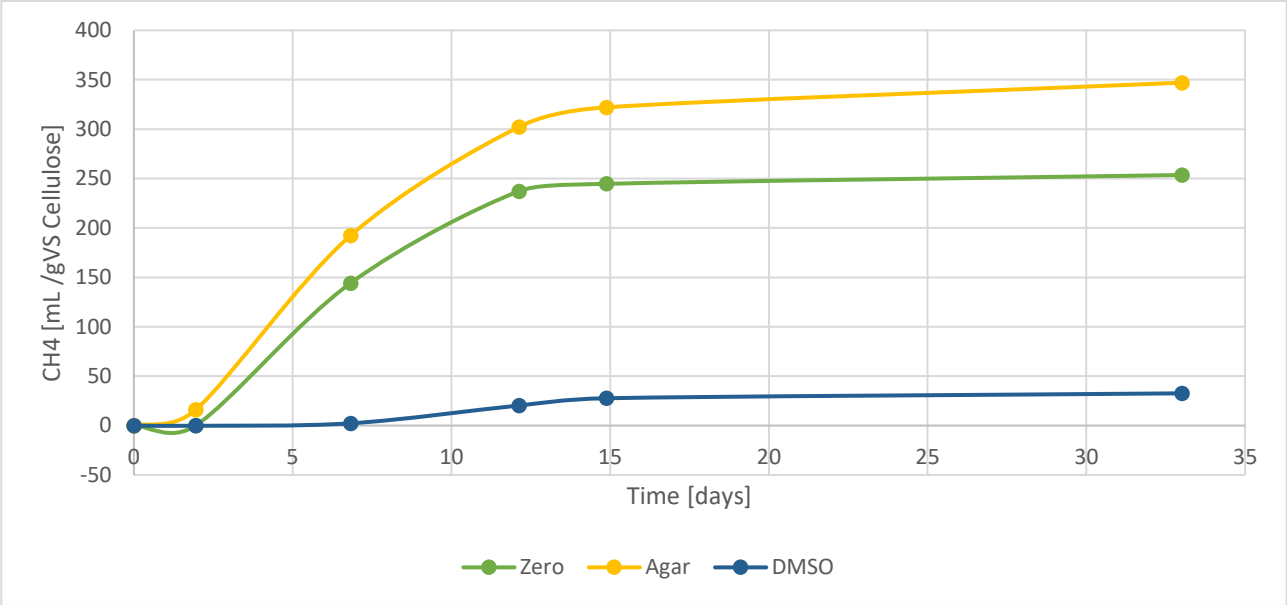


Figure 26 *Cumulative volume of methane produced due to consumption of cellulose per gram of volatile solids in the positive control.*

3.2. Revival performances

Samples treated with agar proved to be the most efficient in preserving the inoculum. They had the shortest lag phase and the highest methane production. Even if this strategy seems promising, some drawbacks came up during the analysis. The production of methane is not blocked during the preservation time, as expected, on the contrary the percentage of methane in the headspace biogas grows faster in samples with agar than in the ones with no gel, meaning that agar is used as substrate to carry on the bacterial growth also at 20°C. This assumption is confirmed by **Figure 27**, where time before zero represents the percentage of methane detected during the storage, after zero the values during incubation at 37±1 °C. The production rate during storage is higher in samples with agar. In the references, the percentage of methane in biogas reached at the end of the BMP test is almost the same for Zero 20°C and Agar 20°C. This happens because when cellulose is added, the necessary quantity of carbon for bacterial growth is saturated and the anaerobic digestion process is carried out until the final stages, so biogas reach its typical composition.

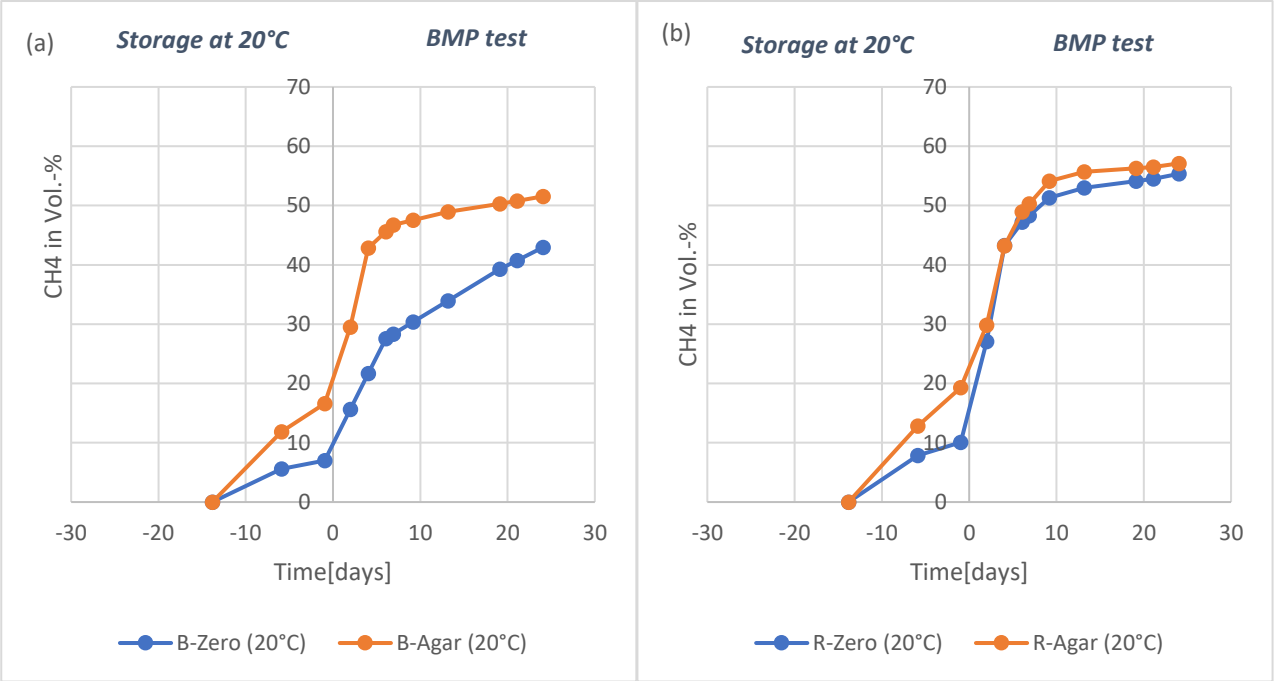


Figure 27 *Percentage of methane in the headspace biogas produced in zero (20°C) and agar samples during the storage and the BMP revival test, for blanks (a) and references (b).*

In **Figure 28**, the cumulative quantity of methane produced during the BMP test is shown. The higher volume of methane produced both in blanks and references treated with agar confirm again the hypothesis that this substance is used as source of carbon by the methanogenic culture.

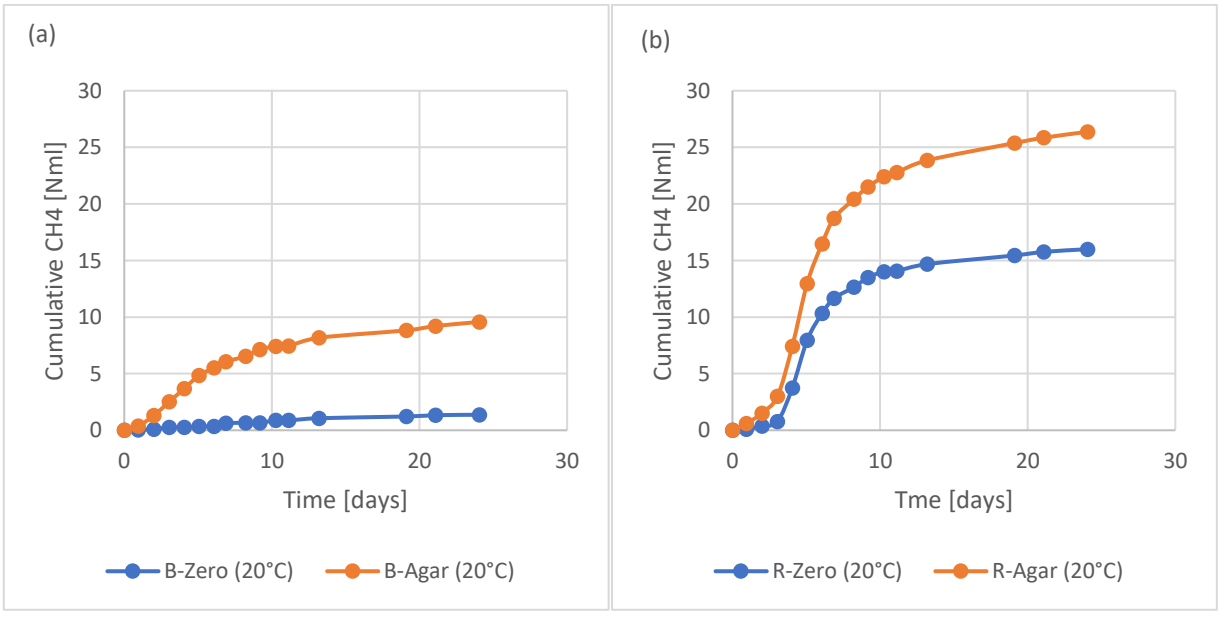


Figure 28 *Cumulative methane produced during the BMP revival test in zero (20°C) and agar samples, for blanks (a) and references (b).*

As aforementioned, for samples containing DMSO, the liquid phase was removed and replaced with BA medium. Even with this additional step, the residual presence of DMSO inhibited the process causing revival performances worse than samples stored at -20°C without cryoprotectant as **Figure 29** shows. Considering the total biogas volume in blanks (**Figure 30**), the production seems almost the same for B-DMSO and B-zero (-20°C); in this case it's important to consider that the maximum methane percentage in B-DMSO is 20%, against the 50% reached by B-zero (-20°C) (**Figure 31**).

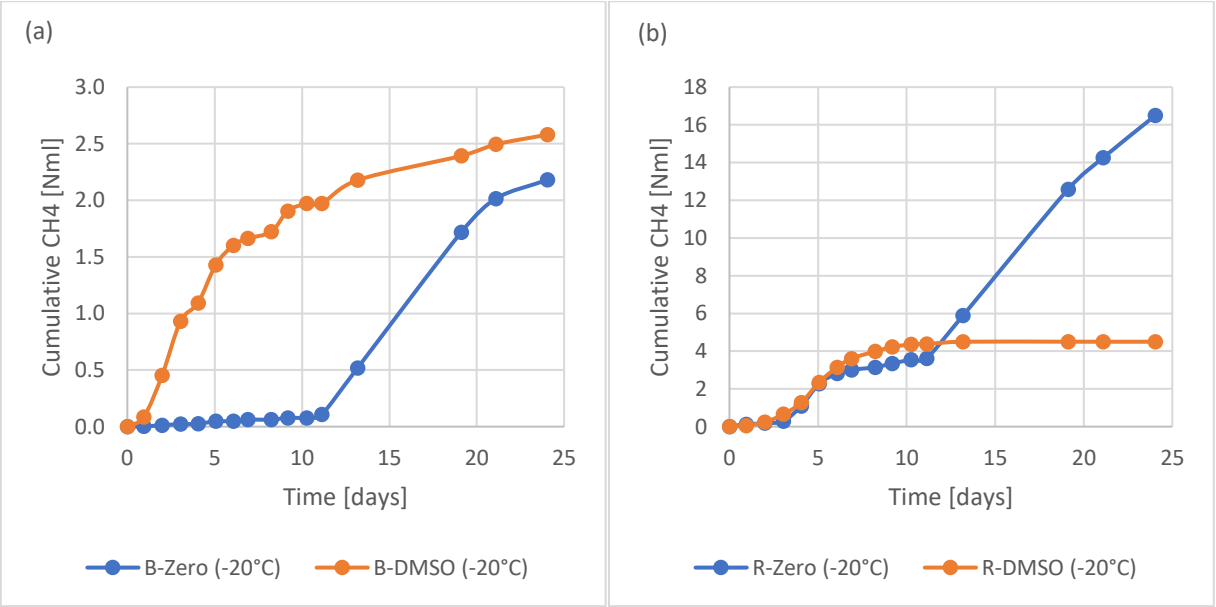


Figure 29 *Cumulative methane production in zero (-20°C) and DMSO samples during the BMP revival test, for blanks (a) and references (b).*

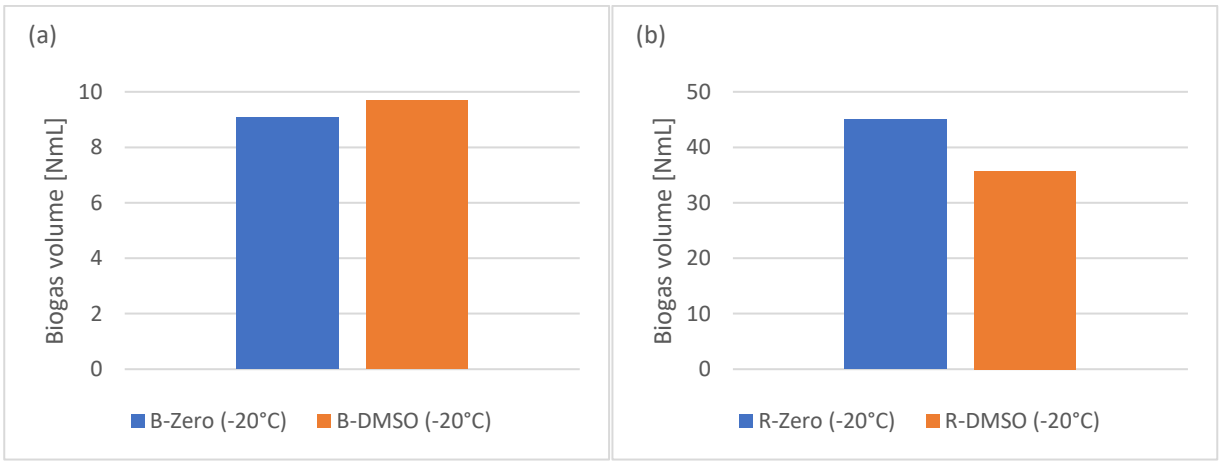


Figure 30 *Total volume of biogas produced during the BMP revival test in zero (-20°C) and DMSO samples, for blanks (a) and references (b).*

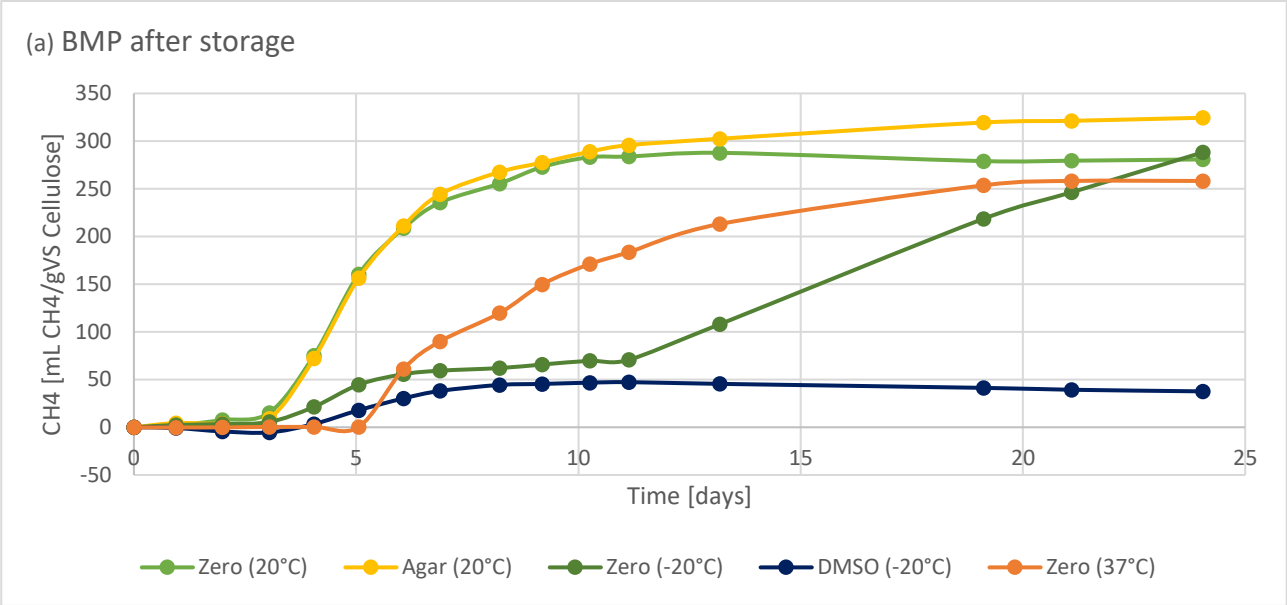
The biogas composition in the headspace at the last measurement for all the strategy is reported in **Figure 31**. Additionally, a sample containing just inoculum was analysed (ZERO 37°C). This sample have been stored for 14 days at 37±1°C without any additional substance. The aim of this analysis is to verify the efficiency of preservation comparing the performance of inoculum when the anaerobic digestion process is not interrupted and can run in ideal conditions.

In R-Zero (37°C), the percentage of methane is much higher than in other samples as in this case the methanogenic culture had more time to reach the final stages of anaerobic digestion, when methane production takes place. The percentage of methane is higher in all the references as the additional substrate enhance the anaerobic digestion.

Figure 31 *Percentage of methane and carbon dioxide in biogas from MBR headspace produced during BMP revival test in blank (a) and reference (b) samples.*

Finally, in **Figure 32** (a), it is reported the comparison between cumulative volume of methane produced by substrate consumption for all the methodologies. Samples treated with agar and samples stored at 20°C without gel have the shortest lag phase but the inoculum preserved with agar reaches

a slightly higher production of methane. The samples of inoculum stored at 37.5 °C produce some methane as well, but the reactivation takes almost the double of time respect to agar strategy. Inoculum stored at -20°C, after 11 days of low activity, started increasing quickly its production rate, reaching on the 24th day the cumulative volume produced by Zero (20°C); this is probably because 14 days at -20°C without cryoprotectant don't cause irreversible damages to the cellular membrane, so that after 11 days of incubation, the cells can restore their usual degradation activity. **Figure 32** (b) is reported to compare the results coming from the positive control and the BMP after storage. The BMP after storage was shut down even if the methane production of Zero (-20°C) was still increasing rapidly because of time limitation. However, in the positive control the Agar samples production at the 20th day was just slightly more than in the BMP after storage, meaning that the preservation period had little impact on the methanogenic community. Zero (20°C) had a production of biogas higher than samples Zero without storage, around 30 NmlCH4/gVS. The mechanism that caused the difference is unknown, but it could be due to operative inaccuracies.



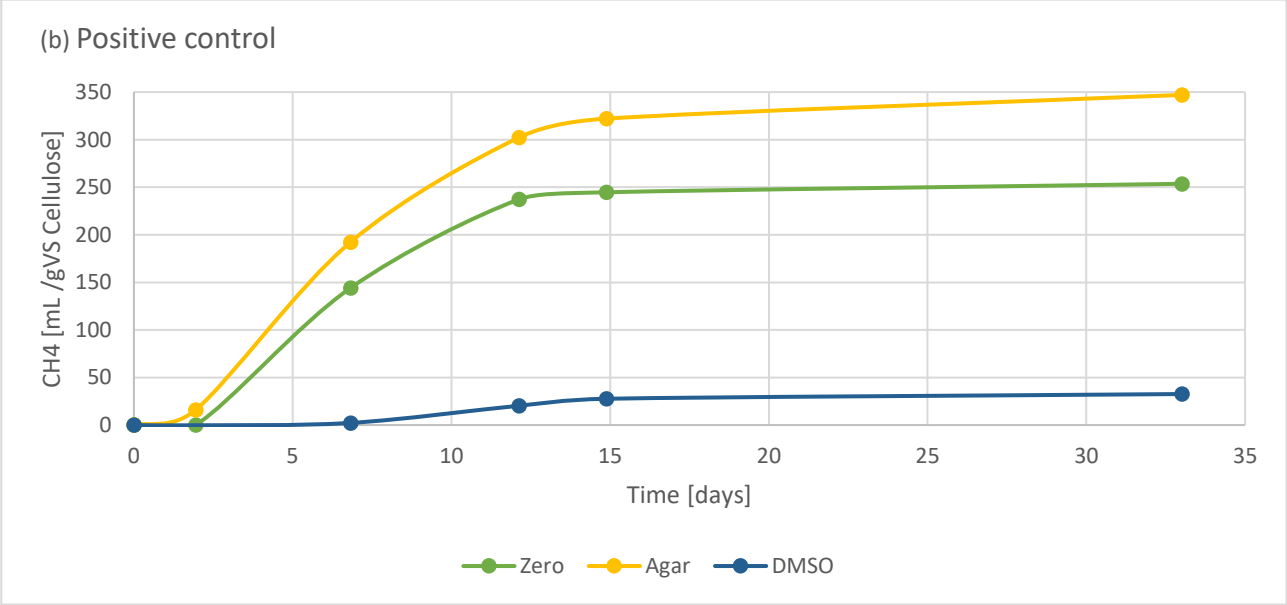


Figure 32 (a) *Cumulative volume of methane produced due to consumption of cellulose per gram of volatile solids in the BMP revival test; (b) cumulative volume of methane produced due to consumption of cellulose per gram of volatile solids in the positive control.*

3.3. Effect of DMSO

Considering the results obtained in the BMP revival test, the removal of DMSO through centrifugation is not enough to completely recover the inhibition. This assumption was confirmed by the toxicity assay, as it is deducible from "no DMSO" samples in **Figure 33**, the removal of DMSO improves a lot the revival, but just the reference sample where DMSO was removed could reach 46.396% of methane in biogas after 25 days and showed an increasing quantity of methane during the process (**Figure 34**). On the other side, blanks without DMSO decreased the quantity of methane

in biogas over time as it is visible. In **Figure 35** the cumulative volume of methane produced by cellulose degradation is reported. The samples with DMSO have negative values because the production of methane in blanks was higher than in reference, as it's visible in **Figure 34**. Samples where DMSO was removed showed better revival, but they could not reach the normal production of methane that for cellulose is between 340 and 395 NmLCH4 gVS (Holliger et al., 2021).

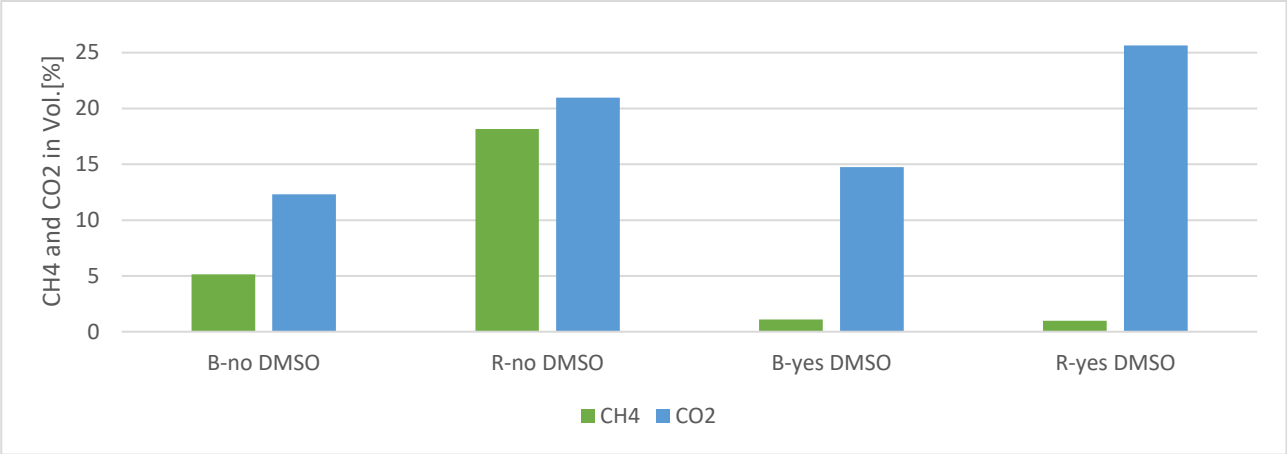


Figure 33 *Percentage of methane and carbon dioxide in biogas from MBR headspace produced during DMSO toxicity BMP test in blank and reference samples.*

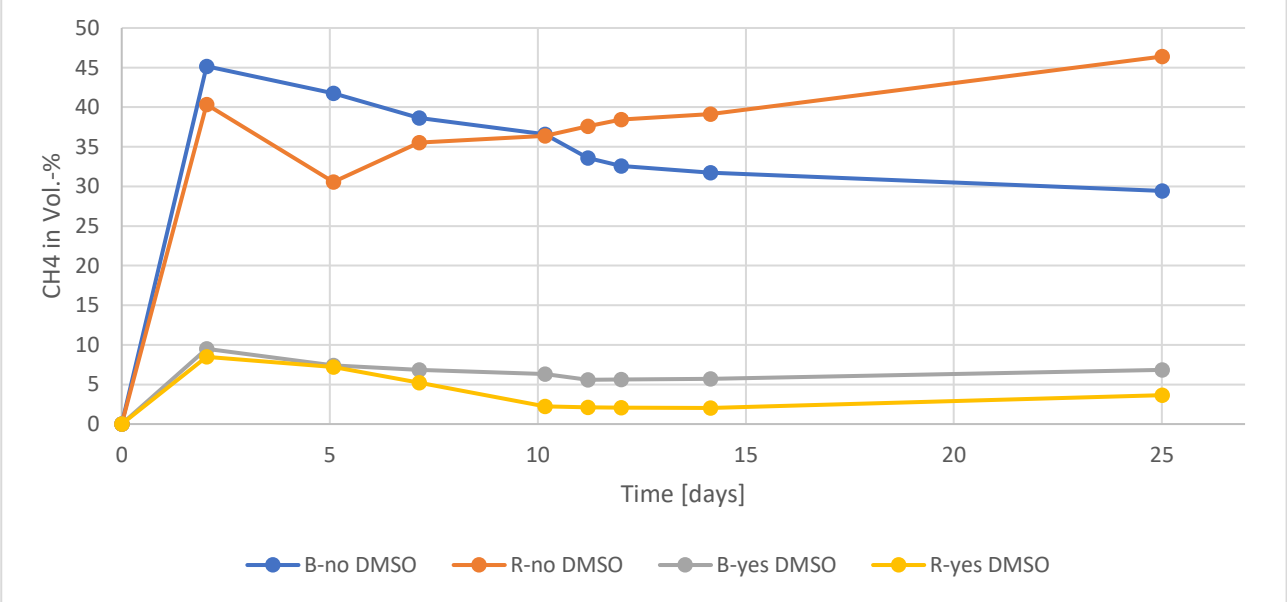


Figure 34 *Percentage of methane in the biogas produced in samples containing DMSO and samples where DMSO was removed by centrifugation, DMSO toxicity BMP test, for blanks and references.*

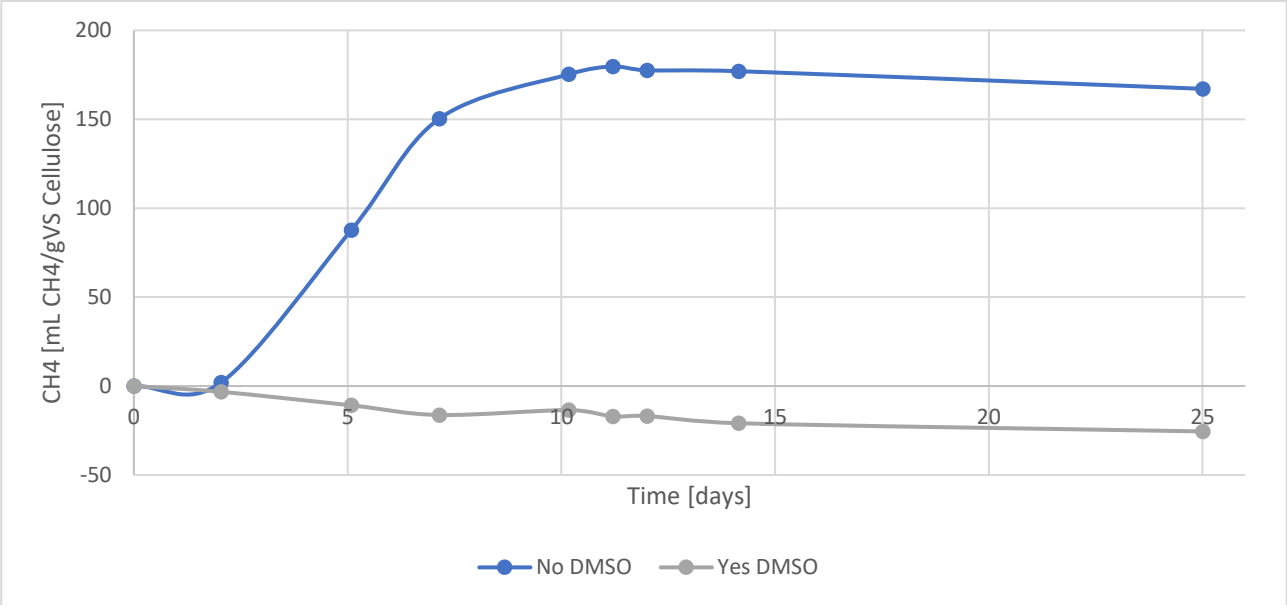


Figure 35 *Cumulative volume of methane produced due to consumption of cellulose per gram of volatile solids in the DMSO toxicity BMP test.*

4. Conclusion

This study provides a first insight of methanogenic consortia preservation through DMSO and agar gel and their revival for BMP testing. The results showed that agar gel represents a good carrier for 14 days storage at 20°C. Specifically, this strategy produces short lag phase (around 3 days) and the highest methane production compared to the other methodologies. On the other side, it is utilized as substrate both during the preservation and the BMP test, inducing an overestimation of the methane volume produced due to consumption of cellulose, so uncertainties in the performances of the bacterial inoculum and efficiency of the substrate. Moreover, further investigation about the effectiveness of this process for long term storage should be carried out, even if promising results were gotten by Yan et al., 2020. When precisely quantified the contribution of agar gel to the methane production per gVS, this strategy could be applied for short term preservation.

DMSO turned out to not be suitable for BMP testing. The reason probably lies in its ability to induce nonlamellar structures in phospholipids and to enhance membrane permeability, that cause damages to biomembranes at physiological temperature, as suggested by previous studies (Hoefman et al., 2012) (Soltys et al., 2012) (Yu & Quinn, 1994).

Other cryoprotectants should be tested before assessing the freezing inadequacy for inoculum preservation, as the state of dormancy induced could allow longer preservation period than through agar gel. Anyways, it is undeniable that the transport of inoculum preserved with agar is much easier

than the transport of frozen inoculum, as refrigeration and freezing may cause high expenses and difficulties for large scale distribution.

It is necessary to point out that considering the standardised protocols proposed by Holliger et al., 2021, the results in this study cannot be considered acceptable to validate the BMP test carried out as from positive control there was a methane production of the inoculum <85% of the theoretical BMP (for cellulose: <340 $NmLCH_4/gVS$). This is true also for the BMP after storage, as shown in **Figure 32** (a), no curve reaches a value of methane production higher than 340 $NmLCH_4/gVS$. There could be many causes related to this result. The first deals with an operative step during the experimental set up. When cellulose was poured inside the MBRs, around 0.5% of the weighed quantity remained on the plastic vessel because of the electric charge of cellulose particles, se the quantity of gVS coming from substrate was overestimated. Secondly, the inoculum could be damaged because of too much intrusion of air during the preparation. Finally, it could be due to unknown changes in the WWTP digester, so that inoculum characteristics may be variated.

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THIRD PART

Annex

THIRD PART

ANNEX A: Tables related to the pre-test on MBRs materials

Table 17 *Volumes of biogas measured during the pre-test on MBRs materials.*

				Glass [ml]						Teflon[ml]					
				Blank			Ref			Blank			Ref		
Date	Time	T [°C]	P [hPa]	1	2	3	4	5	6	7	8	9	10	11	12
09/11/21	17:21	37.8	1026	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10/11/21	13:23	37.6	1024	7.30	7.40	7.30	8.10	7.50	7.60	7.20	7.20	7.50	7.60	7.40	7.60
10/11/21	17:51	37.6	1024	1.10	1.30	1.30	1.40	1.40	1.40	1.10	1.40	1.30	1.40	1.50	1.40
11/11/21	9:20	37.8	1024	2.50	3.00	2.90	5.00	4.50	4.00	2.70	2.60	2.80	3.50	3.50	3.50
11/11/21	17:19	37.6	1023	1.00	1.00	1.00	3.80	3.40	2.90	1.10	1.00	1.00	2.00	2.20	2.30
12/11/21	12:22	37.4	1019	1.60	2.50	2.70	15.50	13.80	13.50	2.20	2.10	2.00	11.30	9.70	9.50
13/11/21	13:00	37.6	1013	2.40	2.70	2.70	17.50	20.20	20.90	2.00	2.40	2.40	18.60	18.70	19.00
14/11/21	11:30	37.6	1026	1.70	1.80	1.70	8.40	9.80	10.00	1.20	1.30	1.30	9.70	10.30	10.70
15/11/21	11:50	37.6	1030	1.70	1.70	1.60	5.40	5.50	5.80	1.40	1.30	1.20	5.30	5.90	6.00
16/11/21	9:27	37.5	1025	1.50	1.50	1.40	3.50	4.00	4.00	1.00	1.30	1.20	3.30	3.60	3.70
17/11/21	11:33	37.9	1017	2.00	1.60	1.70	4.20	4.00	4.00	1.60	1.40	1.40	3.50	3.40	3.40
19/11/21	12:00	37.7	1023	1.90	2.00	2.10	3.80	4.80	4.70	1.20	1.40	1.50	2.80	3.30	3.40
22/11/21	10:32	37.5	1026	2.40	2.60	2.50	4.30	4.40	4.50	1.70	1.90	1.80	2.90	3.00	3.00
23/11/21	11:20	37.5	1027	1.00	1.10	1.10	1.60	1.80	1.80	0.80	0.90	0.80	0.90	1.00	1.00
26/11/21	14:22	37.7	993	3.10	3.20	3.00	4.20	3.80	3.60	2.30	2.10	2.30	2.60	2.00	2.20
29/11/21	12:30	37.7	1005	1.00	1.20	1.20	2.40	1.40	1.50	0.30	0.40	0.40	0.00	0.50	0.40
01/12/21	12:09	37.6	984	1.5	1.4	1.4	1.8	1.6	1.4	1.2	0.9	0.9	1.2	0.7	0.7

Table 17

				PPCO [ml]				
				Blank		Ref		
Date	**Time**	**T [°C]**	**P [hPa]**	**13**	**15**	**16**	**17**	**18**
09/11/21	17:21	37.8	1026	0.00	0.00	0.00	0.00	0.00
10/11/21	13:23	37.6	1024	6.80	7.40	7.30	7.40	7.40
10/11/21	17:51	37.6	1024	1.10	0.90	1.30	1.40	1.40
11/11/21	9:20	37.8	1024	2.50	3.00	3.30	3.10	3.10
11/11/21	17:19	37.6	1023	1.20	1.20	2.00	2.30	2.20
12/11/21	12:22	37.4	1019	2.20	2.10	8.90	9.40	9.60
13/11/21	13:00	37.6	1013	2.00	2.20	18.50	18.30	19.00
14/11/21	11:30	37.6	1026	1.20	1.30	10.20	10.20	10.20
15/11/21	11:50	37.6	1030	1.20	1.00	5.50	5.50	5.30
16/11/21	9:27	37.5	1025	1.00	1.00	3.00	3.00	3.30
17/11/21	11:33	37.9	1017	1.20	1.20	3.20	2.90	3.00
19/11/21	12:00	37.7	1023	1.20	1.20	2.60	2.70	3.00
22/11/21	10:32	37.5	1026	1.00	1.00	1.60	1.90	1.90
23/11/21	11:20	37.5	1027	0.30	0.60	0.50	0.60	0.90
26/11/21	14:22	37.7	993	1.70	1.50	1.60	1.50	1.10
29/11/21	12:30	37.7	1005	0.00	0.00	0.00	0.00	0.00
01/12/21	12:09	37.6	984	1.2	0.4	1.2	0	0.3

Table 18 *Evolution of the biogas composition during the pre-test on MBRs materials.*

		GLASS-Blank-1			GLASS-Reference-4		
Date	**Time in days**	**B-G-CH4**	**B-G-CO2**	**Real CH4 produced %**	**R-G-CH4**	**R-G-CO2**	**Real CH4 produced %**
09/11/2021	0.000	0.000	0.000	0.000	0.000	0.000	0.000
10/11/2021	1.000	10.032	10.639	48.534	9.900	11.163	47.001
12/11/2021	3.000	27.978	16.414	63.025	40.799	28.316	59.030
15/11/2021	6.000	36.621	19.965	64.718	48.128	40.435	54.343
17/11/2021	8.000	38.920	21.736	64.165	50.034	39.775	55.712
19/11/2021	10.000	41.759	21.969	65.527	53.500	39.432	57.569
26/11/2021	17.000	45.088	23.770	65.480	54.406	38.977	58.261
01/12/2021	22.000	48.050	25.785	65.078	55.009	38.870	58.595

FEP-Blank-7			FEP-Reference-10			PPCO-Blank-13			PPCO-Reference-16		
B-T-CH4	**B-T-CO2**	**Real CH4 produced %**	**R-T-CH4**	**R-T-CO2**	**Real CH4 produced %**	**B-P-CH4**	**B-P-CO2**	**Real CH4 produced %**	**R-P-CH4**	**R-P-CO2**	**Real CH4 produced %**
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9.323	9.863	48.593	8.272	9.946	45.405	7.826	9.836	44.310	8.494	9.782	46.479
23.283	14.026	62.405	32.760	21.096	60.829	24.301	14.829	62.103	31.896	20.034	61.421
29.974	15.424	66.026	45.461	33.573	57.521	31.372	15.881	66.392	45.795	34.836	56.796
32.098	15.871	66.914	48.570	32.226	60.114	33.296	16.122	67.377	49.075	33.597	59.361
34.400	15.345	69.153	51.859	29.394	63.824	34.848	15.611	69.061	50.620	29.934	62.839
36.239	14.153	71.913	53.521	22.336	70.555	37.034	14.843	71.387	55.626	24.598	69.338
36.860	13.495	73.200	52.902	19.188	73.383	37.570	14.514	72.134	55.327	21.517	72.000

ANNEX B: Tables related to the final experiment

Table 19 *Volumes of bigas measured during the Positive control.*

				BLANK [ml]						REFERENCE [ml]					
				ZERO		AGAR		DMSO		ZERO		AGAR		DMSO	
Date	Time	T [°C]	P [hPa]	1	2	5	6	9	10	13	14	17	18	21	22
28/01/2022	16:20	37.2	1030	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29/01/2022	12:43	37.4	1012	1.40	1.40	2.30	2.20	1.40	1.50	1.00	0.90	4.30	5.40	1.40	1.40
30/01/2022	15:05	37.5	1021	1.00	0.50	1.00	0.40	1.00	0.20	1.00	1.10	1.00	0.00	1.00	0.00
31/01/2022	15:38	37.2	1006	0.40	1.00	0.80	0.90	0.70	0.70	0.90	0.80	1.00	2.10	0.80	0.80
01/02/2022	14:08	37.5	1001	0.70	0.50	1.10	0.90	0.50	0.30	0.30	0.50	2.50	1.60	0.50	0.50
02/02/2022	11:14	37.3	1012	0.00	0.00	1.60	1.30	0.00	0.00	1.50	2.40	8.60	4.80	0.00	0.00
03/02/2022	10:51	37.3	1014	0.20	0.00	2.40	2.00	0.50	0.50	8.80	9.80	13.50	11.60	0.60	0.50
04/02/2022	12:04	37.7	1005	0.40	0.40	3.10	2.80	0.80	0.90	10.50	10.50	10.80	12.70	1.50	1.20
05/02/2022	16:55	37.5	1013	0.20	0.00	3.80	3.60	0.90	0.90	7.30	6.60	7.10	9.20	1.80	1.00
06/02/2022	12:21	37.6	993	0.80	0.90	3.60	3.50	1.00	1.20	3.20	2.40	3.20	5.90	2.20	2.00
07/02/2022	11:32	37.5	1015	0.00	0.00	0.90	1.00	0.00	0.00	0.50	0.50	2.00	4.60	1.30	1.00
08/02/2022	10:54	37.7	1019	0.00	0.00	1.10	1.40	0.20	0.10	0.80	0.90	2.50	4.20	1.80	1.60
09/02/2022	19:20	37.5	1021	0.00	0.10	1.10	1.20	0.60	0.30	1.10	1.10	4.20	3.70	2.50	2.60
10/02/2022	14:30	37.5	1016	0.50	0.30	0.80	0.90	0.30	0.50	0.60	0.80	2.30	2.00	1.50	2.00
11/02/2022	12:06	37.5	1022	0.00	0.00	0.50	0.50	0.30	0.10	0.40	0.20	1.00	1.20	0.90	1.40
12/02/2022	13:26	37.4	1028	0.00	0.00	1.00	0.30	0.00	0.00	0.30	0.20	1.00	0.80	1.10	2.20
13/02/2022	12:50	37.5	1013	0.50	0.50	1.00	1.00	0.60	0.60	0.80	0.70	1.50	1.80	1.10	2.30
14/02/2022	14:16	37.4	999	0.40	0.40	0.70	0.80	0.60	0.60	0.60	0.60	1.40	1.30	1.00	2.60
16/02/2022	14:30	37.5	990	0.50	0.50	1.50	1.40	1.00	1.00	1.00	0.80	2.20	2.00	1.40	3.10
18/02/2022	10:41	37	1000	0.00	0.00	0.50	0.50	0.30	0.20	0.10	0.00	0.80	0.50	0.40	1.90
21/02/2022	19:16	37.6	997	0.60	0.50	0.50	1.10	0.60	0.60	0.60	1.00	1.60	1.90	1.00	2.30

24/02/2022	17:41	37.5	1000	0.20	0.40	1.20	1.60	0.40	0.30	0.80	0.70	1.20	1.10	0.70	1.20
02/03/2022	16:30	37.5	1025	1.00	0.70	1.00	1.10	0.50	0.00	0.70	0.30	1.10	0.70	0.70	0.30

Table 20 *Evolution of biogas composition during the Positive control.*

		B-zero-1			**B-agar-5**		
Date	**Days**	**B-zero-CH4**	**B-zero-CO2**	**Real CH4 produced %**	**B-agar-CH4**	**B-agar-CO2**	**Real CH4 produced %**
30/01/2022	1.95	2.987	5.014	37.329	4.759	5.400	46.845
04/02/2022	6.82	5.539	6.037	41.284	16.928	17.708	48.873
09/02/2022	12.13	8.316	7.150	45.588	30.416	24.301	55.587
12/02/2022	14.88	9.758	7.729	47.823	32.533	25.425	56.132
02/03/2022	33.01	19.250	11.534	62.533	39.028	28.579	57.728

B-dmso-9			**R-zero-13**			**R-agar-17**			**R-dmso-21**		
B-dmso-CH4	**B-dmso-CO2**	**Real CH4 produced %**	**R-zero-CH4**	**R-zero-CO2**	**Real CH4 produced %**	**R-agar-CH4**	**R-agar-CO2**	**Real CH4 produced %**	**R-dmso-CH4**	**R-dmso-CO2**	**Real CH4 produced %**
0.621	5.652	9.898	3.820	5.628	40.435	4.787	6.058	44.140	0.719	6.440	10.042
1.065	8.710	10.238	22.615	29.474	43.417	28.500	38.052	42.824	2.919	17.406	9.637
1.547	12.037	10.607	36.086	32.592	52.544	43.912	37.713	53.798	5.313	29.336	15.333
1.798	13.765	10.799	37.038	32.439	53.255	46.007	36.179	55.979	4.545	36.412	11.097
3.448	25.138	12.062	43.299	31.433	57.939	51.272	36.671	58.301	3.892	48.291	7.458

Table 21 *Volumes of biogas measured during the BMP test for stored samples.*

				1	2	5	6	7	11	12	15	16	17	21
Date	**Time**	**T [°C]**	**P [hPa]**	**B-zero-1 (20°C)**	**B-zero-2 (20°C)**	**B-agar-5**	**B-agar-6**	**B-agar-7**	**R-zero-11 (20°C)**	**R-zero-12 (20°C)**	**R-agar-15**	**R-agar-16**	**R-agar-17**	**B-inoculum-21**
11/02/22	13:43	37.5	1023	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12/02/22	12:20	37.5	1029	0.70	0.60	3.50	2.90	3.00	0.80	1.00	1.90	6.70	6.70	4.60
13/02/22	13:26	37.5	1012	0.50	0.50	3.80	3.70	4.00	1.50	1.10	3.50	3.50	4.00	0.90
14/02/22	14:46	37.5	1000	0.90	1.00	4.00	4.00	4.40	1.60	1.10	5.00	4.90	5.10	0.50
15/02/22	14:56	37.6	1008	0.10	0.10	3.40	2.60	3.90	9.40	7.30	13.00	12.00	12.50	0.10
16/02/22	15:07	37.5	990	0.10	0.70	3.20	2.90	3.80	11.60	11.60	15.60	15.50	15.80	-
17/02/22	15:17	37.4	997	-	-	1.70	1.80	1.90	5.90	6.50	8.30	9.20	9.00	0.30
18/02/22	10:59	37.1	1000	2.00	0.40	1.30	1.60	1.40	2.90	3.90	5.30	5.70	5.60	0.30
19/02/22	19:10	37.6	1009	0.10	0.20	1.10	1.50	1.20	2.00	2.80	4.90	3.30	3.60	-
20/02/22	18:07	37.5	995	-	-	1.30	1.90	1.40	1.70	2.50	3.10	2.50	1.90	0.10
21/02/22	19:52	37.5	998	0.90	0.80	0.60	1.00	0.60	1.20	1.20	2.20	1.70	2.10	0.10
22/02/22	16:53	37.5	1009	0.00	0.00	0.20	0.00	0.10	0.00	0.30	1.10	0.80	0.70	0.00
24/02/22	18:02	37.5	1000	0.70	0.70	1.60	2.00	1.80	1.30	1.50	2.60	2.40	2.00	0.00
02/03/22	16:29	37.5	1025	0.70	0.20	1.00	1.90	1.80	1.80	1.60	3.60	3.40	2.80	-0.50
04/03/22	15:54	37.6	1025	0.30	0.40	0.60	1.10	1.00	0.70	0.70	1.00	1.00	1.00	0.00
07/03/22	14:48	37.5	1027	0.00	0.20	0.70	0.90	0.90	0.40	0.60	1.20	1.10	1.00	0.00

Table 21

22	25	26	29	30	33	34	35	39	40	43	44	45
B-inoculum-22	**R-inoculum-25**	**R-inoculum-26**	**B-zero-29 (-20°C)**	**B-zero-30 (-20°C)**	**B-DMSO-P-33**	**B-DMSO-34**	**B-DMSO-35**	**R-zero-39 (-20°C)**	**R-zero-40 (-20°C)**	**R-DMSO-43**	**R-DMSO-44**	**R-DMSO-45**
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.10	4.50	0.90	0.00	0.70	1.00	0.80	1.00	5.00	5.00	1.00	0.60	1.00
0.70	0.90	0.60	1.00	0.70	2.10	1.60	2.10	1.20	1.10	1.60	1.60	1.80
0.50	0.60	0.60	0.50	0.50	1.40	2.00	1.60	1.50	1.60	2.30	2.80	2.50
0.10	0.10	0.10	0.10	0.10	0.40	0.80	0.50	6.20	6.30	5.70	3.80	4.50
-	-	-	0.60	0.40	1.10	1.30	1.20	7.20	6.40	11.10	10.50	13.00
0.20	8.90	14.50	-	-	1.20	0.40	0.30	3.20	2.80	7.80	8.20	9.50
0.30	5.40	4.80	0.20	0.20	0.10	0.30	0.30	1.10	1.00	4.30	4.70	5.60
-	5.90	2.60	-	-	0.20	0.30	0.20	0.70	0.70	3.70	3.90	4.30
0.10	5.50	2.50	0.00	0.30	0.80	0.70	0.80	1.00	1.00	2.40	2.30	2.80
0.10	3.00	2.40	-	-	0.30	0.30	0.30	1.00	0.50	1.60	1.10	1.60
0.00	1.00	1.90	0.20	0.20	0.00	0.00	0.00	0.00	0.50	0.10	0.10	0.60
0.10	2.50	4.00	1.90	2.30	1.10	1.00	1.00	4.70	6.10	1.10	1.00	1.50
-0.30	3.10	4.40	4.60	5.20	1.30	1.10	1.20	13.60	14.50	0.00	0.00	0.00
0.00	0.50	0.40	1.00	0.50	0.80	0.50	0.50	4.00	2.80	0.00	0.00	0.00
0.00	0.00	0.00	0.40	0.40	0.50	0.50	0.60	4.30	4.30	0.00	0.00	0.00

Table 22 *Evolution of biogas composition during BMP test of stored samples.*

		B-zero-1 (20°C)			B-agar-5			R-zero-11 (20°C)			R-agar-15		
Date	**Days**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**
11/02/22	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13/02/2022	1.988	3.525	19.017	15.637	11.084	26.493	29.496	2.919	7.862	27.076	5.795	13.648	29.804
14/02/2022	3.044	-	-	-	-	-	-	-	-	-	-	-	-
16/02/2022	4.051	-	-	-	23.111	30.807	42.863	21.029	27.542	43.295	28.017	36.791	43.231
17/02/2022	5.058	-	-	-	-	-	-	-	-	-	-	-	-
18/02/2022	6.065	7.942	20.873	27.563	-	-	-	29.056	32.489	47.211	37.541	39.187	48.927
19/02/2022	6.886	-	-	-	26.011	29.652	46.729	-	-	-	-	-	-
20/02/2022	8.227	-	-	-	-	-	-	-	-	-	-	-	-
21/02/2022	9.183	-	-	-	-	-	-	34.105	32.371	51.304	44.751	37.942	54.117
22/02/2022	10.256	-	-	-	-	-	-	-	-	-	-	-	-
24/02/2022	11.132	-	-	-	-	-	-	-	-	-	-	-	-
26/02/2022	13.180	-	-	-	31.792	33.199	48.918	35.641	31.622	52.988	46.764	37.207	55.691
02/03/2022	19.115	14.386	22.235	39.282	-	-	-	-	-	-	-	-	-
04/03/2022	21.091	-	-	-	35.279	34.229	50.755	37.932	31.692	54.481	48.988	37.713	56.502
10/03/2022	24.045	16.668	22.148	42.940	36.578	34.393	51.540	39.706	32.025	55.354	50.279	37.767	57.106

Table 22

B-inoculum-21			R-inoculum-25			B-zero-29 (-20°C)			B-DMSO-33			R-zero-39 (-20°C)			R-DMSO-43		
CH4	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	0.497	21.301	2.279	4.446	8.136	35.334	1.173	11.305	9.401	2.179	8.389	20.620
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	5.553	10.384	34.841	9.413	34.205	21.580	5.338	40.661	11.605
2.851	6.363	30.943	12.384	22.301	35.705	1.734	22.332	7.203	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.546	49.533	11.672
4.223	6.947	37.806	28.943	26.065	52.615				5.584	13.368	29.465	12.637	38.313	24.802	7.238	54.647	11.696
-	-	-	-	-	-	4.199	22.904	15.493	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	5.862	16.780	25.890	-	-	-	7.494	56.711	11.672
5.976	8.146	42.318	37.469	23.071	61.891	-	-	-	-	-	-	34.643	32.375	51.692	-	-	-
-	-	-	-	-	-	-	-	-	5.447	19.980	21.423	-	-	-	8.063	53.985	12.995
-	-	-	38.830	18.771	67.412	20.790	23.037	47.437	5.443	21.385	20.288	43.676	30.387	58.971	9.524	54.002	14.992
8.704	8.641	50.182	36.791	14.650	71.520	22.431	22.894	49.489	5.470	23.088	19.153	48.906	29.261	62.566	9.799	49.350	16.566

Table 23 *Volumes of bigas measured during the BMP test for DMSO assay.*

					NO DMSO				**YES DMSO**			
					BLANK		**REFERENCE**		**BLANK**		**REFERENCE**	
Date	**Days**	**Time**	**T [°C]**	**P [hPa]**	**25**	**26**	**29**	**30**	**33**	**34**	**37**	**38**
					B- no dmso - 25	**B- no dmso - 26**	**R- no dmso - 29**	**R- no dmso- P-30**	**B- yes dmso- 33**	**B- yes dmso - 34**	**R- yes dmso- 37**	**R- yes dmso- 38**
10/02/22	0.00	14:17	37.5	1016	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11/02/22	0.92	12:22	37.5	1022	1.80	1.70	2.00	2.80	1.20	1.30	0.90	1.00
12/02/22	2.04	15:12	37.4	1026	1.70	1.50	1.50	1.80	1.00	0.40	1.00	0.50
13/02/22	2.92	12:26	37.4	1013	1.20	1.10	1.50	1.30	0.90	1.00	1.00	1.20
14/02/22	4.01	14:33	37.4	1000	1.10	1.00	12.40	5.20	1.40	1.20	1.30	1.30
15/02/22	5.09	16:20	37.6	1007	1.00	0.00	12.20	11.40	1.00	0.30	1.00	0.50
16/02/22	6.03	15:07	37.5	990	1.00	1.30	8.00	8.50	1.30	1.30	1.70	1.50
17/02/22	7.15	17:50	37.4	1000	1.00	0.10	4.70	5.70	1.00	1.10	2.50	1.40
18/02/22	7.84	10:30	37	1001	0.00	0.30	1.80	2.90	0.60	0.80	2.90	2.30
19/02/22	9.22	19:27	37.6	1009	-	-	0.90	2.20	0.40	0.40	5.70	4.50
20/02/22	10.17	18:25	37.5	995	0.90	0.80	1.00	1.80	1.00	0.90	4.40	3.80
21/02/22	11.20	19:07	37.6	997	0.20	0.20	1.00	0.90	1.00	0.30	2.30	2.80
22/02/22	12.01	14:26	37.4	1012	1.00	0.00	0.00	0.10	0.00	0.00	0.80	1.00
24/02/22	14.15	17:53	37.5	1000	0.4	1.0	0.5	0.50	1	0.50	1.5	1.20
02/03/22	20.15	17:48	37.4	1024	0.10	0.10	-0.5	-0.7	0.10	0.00	0.00	-0.5
07/03/22	25.01	14:29	37.5	1027	0.5	0.10	0	-1.00	0.5	0.00	-1	-1.00

Table 23

		B- no dmso-25			R- no dmso-29			B- yes dmso-33			R- yes dmso-37		
Date	**Days**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**	**CH4**	**CO2**	**Real CH4 produced %**
10/02/22	0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
12/02/22	2.04	4.493	5.455	45.165	4.059	5.999	40.358	0.590	5.619	9.496	0.566	6.101	8.483
15/02/22	5.09	5.825	8.123	41.763	13.478	30.632	30.555	0.731	9.165	7.386	0.758	9.780	7.191
17/02/22	7.15	5.871	9.325	38.633	19.622	35.573	35.550	0.852	11.588	6.847	0.848	15.436	5.206
20/02/22	10.17	-	-	-	-	-	-	-	-	-	0.781	34.025	2.245
21/02/22	11.20	-	-	-	21.238	35.264	37.588	0.862	14.595	5.576	-	-	-
22/02/22	12.01	5.581	11.547	32.583	-	-	-	-	-	-	-	-	-
24/02/22	14.15	-	-	-	20.009	31.146	39.115	0.939	15.539	5.696	0.809	38.881	2.039
07/03/22	25.01	5.140	12.323	29.433	18.157	20.978	46.396	1.082	14.760	6.830	0.976	25.649	3.664