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Effect of Endogenous Methane Production: A Step Forward in the Validation of Biochemical Methane Potential (BMP) Tests

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Abstract: This work evaluates the influence of the inoculum type, the pre-consumption of the residual substrate and the ratio of blanks' headspace volume to working volume (Hv Wv^{-1}, 0.6 to 10) on Biochemical Methane Potential (BMP) measurements when methane is monitored by gas chromatography. Different inocula were tested: digested sewage sludge—DSS, granular sludge—GS and fresh dairy manure—DM. Microcrystalline cellulose was used as the substrate. BMP surpassed the maximum theoretical value (BMP_{max} = 414 L kg^{-1}) when methane produced in the blanks was not discounted, showing that degassing cannot stand alone as an alternative to the procedure of discounting the inoculum's background production. Still, when the residual substrate concentration is high (e.g., in DM), degassing is mandatory because methane produced from its digestion will conceal the methane produced from the substrate in the BMP determination. For inocula with a low residual substrate (e.g., GS), short degassing periods are recommended in order to avoid detrimental effects on methanogenic activity. For moderate residual substrate concentrations (e.g., DSS), BMP values closer to BMP_{max} (90–97%) were achieved after degassing and discounting the blanks with lower Hv Wv^{-1}. For higher Hv · Wv^{-1}, less accurate quantification occurred, likely due to error propagation. Proper inoculum pre-incubation time and discounting the methane production from blanks with low Hv Wv^{-1} (adjusted according to the estimated background methane) are essential for accurate BMP determinations.

Keywords: anaerobic biodegradability; biomethane potential; blank assays; degassing; inoculum; digested sewage sludge; granular sludge; dairy manure; residual substrate

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1. Introduction

Biomethane can be produced from the upgrading of biogas, generated by the anaerobic digestion (AD) of organic wastes. Biomethane is an attractive renewable energy source with the potential to displace fossil fuels and contribute to current carbon-neutrality goals, thus leveraging a circular bio-economy [1]. Recent targets were launched by the European Commission, aiming for the production of 35 billion cubic meters (bcm) of biomethane by 2030, and it is estimated that biomethane production in Europe has the potential to reach 95 bcm by 2050 [1,2].

The assessment of the potential methane production from different organic waste is important for the successful operation and optimization of biomethane plants. Biochemical methane potential (BMP) assays are used to determine the ultimate methane production of organic substrates and can also enable the calculation of the time course of the methane production rate [3]. These are essential parameters for the design and economic feasibility assessment of biogas/biomethane plants, thus requiring accurate and reliable determination. In BMP tests, biogas production is usually normalized to standard temperature and pressure conditions (STP, T = 273.15 K and p = 101.33 kPa), and the resulting biogas

potential is generally expressed per mass unit of organic matter, e.g., volatile solids (VS) or chemical oxygen demand (COD), of substrate added. This calculation method eliminates the variability of biomass in terms of moisture and nutrients and allows a basic level of comparison between substrates and different test results. Nevertheless, the existing international standards for BMP assays leave room for different experimental procedures, which is generally reflected in relatively high variability of BMP results and in difficulties in comparing results among different studies [4–8]. Recently, major efforts have been made to identify the parameters responsible for the observed variability [9–14] and to promote the harmonization of the BMP tests [7,15–18]. A new website was established to share detailed guidance on BMP measurement, validation criteria and data processing [18]. The quality of the inoculum [19–21], the gas measurement technique [22–25] and the design of the assays [12,21] are important factors influencing BMP data.

The use of a highly active inoculum containing a wide microbial diversity is mandatory for assuring the presence of all trophic groups and preventing metabolic limitations [7]. Typical inocula used in BMP tests include digested sludge from municipal wastewater treatment plants or animal manure. Granular sludge from industrial bioreactors can be used as well for some types of substrates [4,6]. The selection and storage of the inoculum were shown to influence the rate of methane production, but the effects on BMP were not consistent [12–14,19,20,26,27]. For example, Hafner et al. [13] verified that the inoculum was not a major source of BMP variability, while De Vrieze et al. [19] reported differences in BMP values of up to 2.4 and 1.5 times for liquid pig manure and A-sludge, respectively, when comparing four different inocula.

The influence of the inoculum source on BMP determination has been mainly attributed to the activity and composition of the microbial communities, as well as to the presence or absence of nutrients. However, the residual substrate present in the inoculum may also be an important factor to consider in BMP assays because it varies with the inoculum type, and the endogenous methane production that results from its biodegradation can contribute to overestimations of the methane measurements from the target substrate. Inoculum to substrate ratios (ISr) between 2 and 4 g g^{-1} (in VS) have been recommended to minimize the acidification or inhibition problems [4,7], but larger amounts of inoculum will generate more background methane production. To avoid this problem, it is usual to perform blank assays without the addition of the target substrate, and after, subtract the endogenous methane production from the gross methane production obtained in the assays amended with the substrate. Nevertheless, the possibility of applying alternative strategies, other than discounting the blanks, to overcome the limitations that arise from the background methane production has not been studied in detail. For example, by degassing the inoculum, i.e., pre-incubating it to promote the consumption of the majority of the residual substrate [4,7], less background methane will be formed in the assays with a substrate. This procedure has been applied by several authors (e.g., [5,25]), but the pre-incubation period is generally not monitored, and its impact on BMP determination has not been studied. In the presence of high amounts of the residual substrate, the combination of both strategies may be required, but when the amount of residual substrate is limited (e.g., in the case of granular sludge), pre-incubation for long periods may deteriorate the activity of the inoculum. Therefore, the influence on the BMP results of the residual substrate present in the inoculum used, as well as of its pre-consumption, is still not thoroughly understood and is addressed in the present work.

Another parameter that is not comprehensively studied is the headspace to working volume ratio, which may require careful attention when methane accumulation is monitored by gas chromatography (GC). The methane produced is diluted in the headspace gas, and this effect becomes higher for larger headspace volumes, being critical for blanks, where the amount of methane produced is much lower than in the substrate-amended assays. Small amounts of methane diluted in bigger headspace volumes will lead to very low methane concentrations, likely near the detection limit of the equipment used for quantification. Error propagation can be highly potentiated when multiplying the GC

injected volume (e.g., 0.5 mL) by higher headspace volumes, overestimating the methane produced from the residual substrate and underestimating the BMP of the target substrate. On the other side, the smaller the headspace, the higher the pressure that will be reached. Still, these differences do not represent a significant effect on the solubilization of carbon dioxide in the medium, which could influence the microbial community [28,29]. Thus, blank assays prepared with a smaller headspace volume will theoretically allow more precise and accurate measurement of the relatively low methane produced in these assays. Up to now, little attention has been paid to this effect, which is still insufficiently studied.

This work aims to contribute to the task of improving the quality of BMP measurements by studying the yet unexplored effects of residual substrate, and consequent endogenous methane production, on BMP determinations. The following questions are raised and studied: (i) Is it really necessary to discount the blanks, or can this be replaced by a previous degasification period? (ii) Can/should these two strategies be used together? (iii) What is the effect of using different headspace to working volume ratios (Hv Wv^{-1}) in the blank assays? BMP assays were performed with three different inocula, which were tested without degassing and also after degassing, using microcrystalline cellulose as the model substrate. During the degassing period, the variation of key parameters was followed for each individual inoculum, which is another aspect addressed in this work that has not been studied in detail until now. Additionally, the influence on BMP determination of subtracting the endogenous methane production or using a combination of the two strategies (degassing + discounting the blanks) was studied using a set of blank assays with different Hv Wv^{-1}.

The novelty of this work is based on the evaluation of the effect of the residual substrate in BMP assays, namely through the assessment of the individual and combined effects of degassing the inoculum and discounting the methane production in the blank assay when using different types of inocula. The possibility of applying alternative strategies, other than discounting the blanks, to overcome the limitations that arise from the background methane production has not been studied before and is addressed in this work for the first time. Furthermore, the effect of using different Hv Wv^{-1} ratios in the blank assays when methane is monitored by gas chromatography is also novel and has never been studied before.

2. Materials and Methods

2.1. Inocula

Three different inocula were tested: (i) digested sewage sludge (DSS) from a municipal anaerobic sludge digester, (ii) anaerobic granular sludge (GS) from a brewery wastewater treatment plant and (iii) fresh dairy manure (DM) collected on a dairy farm. After collection, the inocula were stored at 4 °C until use. The inocula were characterized in terms of total and volatile solids content (TS and VS, respectively) and specific methanogenic activity (SMA). Methanogenic activity tests were performed in the presence of acetate (30 mmol L^{-1}) or H_2/CO_2 (80/20 % *v*/*v*, at 100 kPa overpressure) using the pressure transducer technique [30,31]. The methanogenic activity values were corrected for STP conditions, being expressed in mL of methane at STP conditions per amount (g) of inoculum (in VS) and per day (mL g^{-1} d^{-1}), following the guidelines in Hafner et al. [32].

2.2. Degassing Process

For each inoculum (DSS, GS and DM), the consumption of the residual substrate, i.e., the degassing process, was performed in sealed vials at 37 °C. Approximately 20 g of the inoculum (in VS) was added to each vial. The basal medium was made with distilled water and sodium bicarbonate (5 g L^{-1}); resazurin (0.5 g L^{-1}) was used as the redox indicator [31]. The pH was adjusted between 7.0 and 7.2, with NaOH or HCl. Before incubation, the vials' headspace was flushed with N_2/CO_2 (80/20 %, *v*/*v*) at atmospheric pressure, and sodium sulfide (1 mmol L^{-1}) was added as a reducing agent. The cumulative methane production (MP) was measured over 31 days. MP was expressed as volume (L) of methane at STP

conditions per amount (kg) of VS of inoculum added initially (L kg^{-1}). Solids content and SMA in the presence of acetate and H_2/CO_2 were also determined at the end of the assay. Soluble COD (sCOD) and nitrogen ($N_{soluble}$) were also monitored during the incubation. Considering the volume of liquid samples, the working volume and headspace volume were corrected after each sampling event.

In a second trial, a new GS sample was degassed over 5 days (named dGS-5d) following the same procedure, and tested as inoculum in a BMP assay. Additionally, the degassing process of a mixture of GS and DM (50:50 %, *w*/*w*) was also studied over 25 days.

2.3. BMP Assays

Microcrystalline cellulose (Avicel®, average particle size 50 µm, Sigma-Aldrich, St. Louis, MO, USA) was used as the model substrate in the BMP assays (Figure S1 in Supplementary Materials). It is nearly pure cellulose, suitable for anaerobic biodegradation process monitoring since all microbial trophic groups are needed for its biodegradability. The VS content and the COD of cellulose were 1 and (1.2 ± 0.1) g g^{-1}, respectively.

BMP assays of microcrystalline cellulose were performed in serum bottles (100 mL working volume, 600 mL total volume) according to the guidelines defined [4,7]. The exact volume of each bottle was measured by filling it with water and weighing it. Sodium bicarbonate (5 g L^{-1}) was added to guarantee the buffer capacity. Vitamins, micro and macronutrients were prepared and added to the medium according to Angelidaki et al. [4]. Raw inocula, which were not degassed (DSS, GS and DM), as well as the degassed inocula (dDSS, dGS and dDM), were tested at a final concentration (in VS) of 15 g L^{-1}; for dGS-5d, a final concentration of 25 g L^{-1} was used. The inoculum to substrate ratio (ISr), expressed in terms of VS, was 4 g · g^{-1} for all the assays except dGS-5d, for which it was 2 g g^{-1}. After adding the inoculum, substrate and nutrients, the bottles were closed with butyl rubber stoppers and sealed with aluminum crimp caps. The headspace was flushed with a mixture of N_2/CO_2 (80/20 % *v*/*v*) at atmospheric pressure. Sodium sulfide was added at a final concentration of 1 mmol L^{-1} in order to deplete the residual oxygen. For each inoculum tested, blank assays (where no substrate was added) were also prepared, with 100 mL working volume and different Hv Wv^{-1} (0.6, 2.3, 5.0 and 10.1), to assess the precision and accuracy of the detection of the methane produced from the residual substrate. All the assays were performed in duplicate, incubated at 37 °C and manually agitated once a day.

2.4. Methane Quantification and BMP Calculation

The methane quantification method is based on that described by Hansen and coworkers [5]. Information regarding the method's repeatability, reproducibility, detection limit and quality control (based on the results from positive controls performed with cellulose) can be found in [5].

Methane produced during the assays was accumulated in the bottles' headspace. Using a gastight syringe, the biogas present in the headspace was periodically sampled (500 µL) at constant temperature and at the pressure of the assay. Biogas samples were analyzed in a gas chromatograph (GC), and methane was quantified by comparing the area of the peaks in the chromatograms from the sample and from a standard with 40% methane at atmospheric pressure (Equations (1) and (2))

$$n_{sample} = \frac{Area_{sample}}{Area_{standard}} \cdot n_{standard} \quad (1)$$

where n_{sample} = number of moles of methane in the syringe with the sample;
$n_{standard}$ = number of moles in the syringe with the standard;
$Area_{standard}$ = area of the peak in the chromatograms from the standard;
$Area_{sample}$ = area of the peak in the chromatograms from the sample.

$$n_{standard} = \frac{p_{atm} \cdot V_{syringe}}{R \cdot T_{amb}} \cdot x_{CH4} \quad (2)$$

where p_{atm} = atmospheric pressure (101.3 kPa);

$V_{syringe}$ = volume of the standard collected with the gas tight syringe (500 µL);

x_{CH4} = methane molar fraction in the standard (40%);

R = ideal gas constant (8.3145 J mol^{-1} K^{-1});

T_{amb} = ambient temperature.

As referred, the pressure in the syringe with the sample is the same as in the bottle's headspace. Thus, there is no need to consider it for the determination of the number of moles of methane in the vial's headspace, which was calculated by Equation (3).

$$n_{vial}(j) = \frac{V_{headspace}}{V_{syringe}} \cdot n_{sample}(j) + \sum_{i=1}^{j} n_{sample,i} \quad (3)$$

where $n_{\text{vial }(j)}$ = number of moles of methane accumulated in the vial's headspace after j samples;

$V_{headspace}$ = headspace volume (mL) of each vial.

In order to improve the method accuracy, the amount of methane sampled in the previous measurements was added to the n_{vial}, where j represents the number of measurements performed.

The volume of methane accumulated in the vial's headspace was then calculated under STP conditions by Equation (4).

$$V_{CH4} = \frac{n_{vial} \cdot R \cdot T_{STP}}{p_{STP}} \quad (4)$$

where V_{CH4} = volume of methane (L at STP conditions) accumulated in the vial's headspace;

T_{STP} = standard temperature (273.15 K);

p_{STP} = standard pressure (101.3 kPa).

BMP was determined following Equation (5).

$$BMP = \frac{(V_{CH4} - V_{CH4,blank})}{VS_{substrate}} \quad (5)$$

where *BMP* = biochemical methane potential (L kg^{-1});

$V_{CH4,blank}$ = volume of methane (L at STP conditions) produced in the blanks, at the same time point of the sample;

$VS_{substrate}$ = VS amount (kg) of substrate added at the beginning of the assays.

The end of the assay was determined by the criterion defined in Holliger et al. [7], i.e., daily methane production should be below 1% of the net cumulative methane production (subtracting the methane produced in the blank assays), during three consecutive days. Our results were compared and discussed towards the validation criteria for the BMP tests proposed by Holliger and co-workers [7,18]. These include:

(i) relative standard deviation (RSD) of the volume of methane produced in the control assays amended with microcrystalline cellulose should be <6% ($RSD < 6\%$);

(ii) BMP of microcrystalline cellulose should range from 340 to 395 L · kg^{-1} (*BMP_Control*), corresponding to 82–95% of the theoretical maximum BMP value (BMP$_{max}$ = 414 L · kg^{-1}).

Cellulose's BMP values slightly higher than the theoretical maximum may occur due to random errors [15], but the value 395 L kg^{-1} was proposed by Hafner et al. [15] as the limit (~95% of the theoretical), based on the results from a large inter-laboratory study and considering that around 5% of the available electrons from cellulose remain in non-degraded microbial biomass. In this work, we accepted the minimum limit proposed by these authors (i.e., 340 L kg^{-1}, ~82% of the theoretical), but we considered BMP values up to 100% of the theoretical as acceptable.

From the guidelines published by Holliger and co-workers [7], the volume of methane produced in the blanks should be less than 20% of the total methane produced from each

substrate ($CH_4\_Blk < 20\%$), but this criterion was removed [18]. Still, we decided to verify if our results fulfilled this criterion or not.

2.5. Analytical Methods

Methane was analyzed with a GC-2014 Shimadzu ATF model equipped with a Porapak Q column (80–100 mesh) (2 m × 3.75 mm). Nitrogen was used as the carrier gas (30 mL min^{-1}). The detector, injector and oven temperatures were 35, 110 and 220 °C, respectively. TS and VS were measured gravimetrically [33]. Total COD (tCOD), soluble COD (sCOD) and soluble nitrogen ($N_{soluble}$) were determined using cuvette tests (Hach Lange GmbH, Düsseldorf, Germany) and a DR 2800 spectrophotometer (Hach Lange GmbH, Düsseldorf, Germany). For sCOD and $N_{soluble}$ quantification, the samples were centrifuged for 10 min at 21,000× *g* prior to the analysis. Volatile fatty acids (VFA) were determined in a Jasco HPLC (Tokyo, Japan), using an Agilent Hi-Plex H column (300 × 7.7 mm) and a UV detector with a wavelength of 210 nm. The column was maintained at 60 °C. The mobile phase was sulfuric acid (5 mmol·L^{-1}) at a flow rate of 0.6 mL·min^{-1}. Crotonic acid was used as an internal standard.

2.6. Statistical Analysis

The BMP results were compared after a statistical significance analysis by using single factor analysis of variance (ANOVA). Statistical significance was established at the *p*-value < 0.05.

3. Results and Discussion

3.1. Degassing Process

The residual substrate present in the inoculum can influence the methane production pattern from a target substrate during a BMP test. A strategy to overcome this interference may be the degassing of the inoculum, which reduces the amount of residual substrate and the associated endogenous methane production. In the guidelines published by Holliger et al. [7], it is recommended that the inoculum should have a low endogenous methane production, and the application of a pre-incubation period is proposed, if necessary, to decrease the amount of residual substrate. This procedure has been applied by several authors (e.g., [5,25,29]), but the pre-incubation period is generally not monitored, and its impact on BMP determination has not been comprehensively assessed. Here, three different inocula—DSS, GS and DM—were monitored during the degassing process, in terms of methane production, sCOD and $N_{soluble}$ concentration (Figure 1). Total and volatile solids and the SMA activity of the inocula, before and after the degassing process, are presented in Table 1.

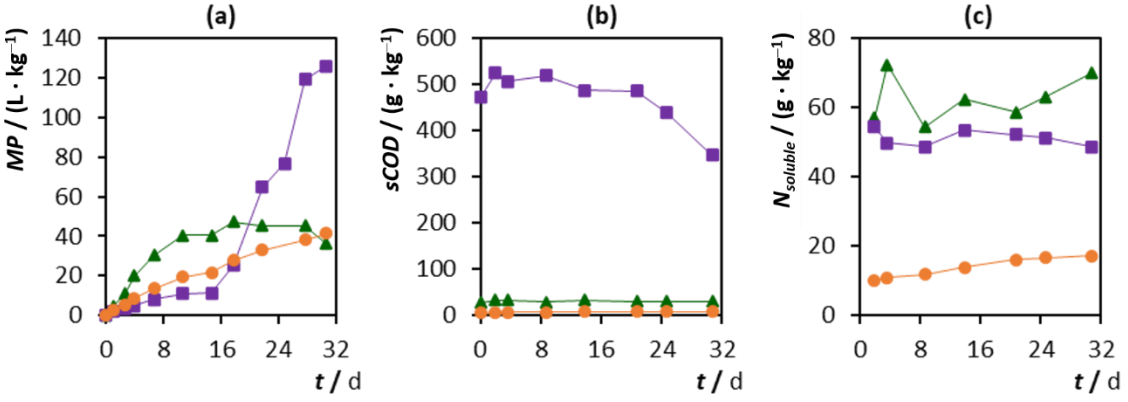

Figure 1. (**a**) Cumulative methane production (MP), (**b**) soluble COD (sCOD) and (**c**) $N_{soluble}$ measured over the degassing process of the digested sewage sludge (DSS, ▲), granular sludge (GS, ●) and fresh dairy manure (DM, ■). All parameters are expressed per kg of VS of inoculum added in the beginning of the assay.

Table 1. Solids content and SMA of DSS, GS and DM, before and after the degassing process (dDSS, dGS and dDM).

Inoculum	$\frac{TS}{g \cdot L^{-1}}$	$\frac{VS}{g \cdot L^{-1}}$	$\frac{SAA}{mL \cdot g^{-1} \cdot d^{-1}}$	$\frac{SHA}{mL \cdot g^{-1} \cdot d^{-1}}$
DSS	25.2 ± 0.3	17.1 ± 0.2	52 ± 4	267 ± 10
dDSS	22.2 ± 0.7	14.8 ± 0.5	<10	91 ± 4
GS	97.3 ± 2.6	88.8 ± 2.4	216 ± 15	497 ± 32
dGS	89.8 ± 1.0	81.9 ± 1.0	112 ± 1	528 ± 36
DM	20.6 ± 0.2	15.6 ± 0.1	<10	206 ± 5
dDM	10.2 ± 0.1	6.6 ± 0.0	49 ± 3	101 ± 15

SAA—Specific acetoclastic methanogenic activity; SHA—Specific hydrogenotrophic methanogenic activity.

Despite having similar sCOD concentrations, DSS and GS presented two distinct profiles of cumulative methane production (Figure 1a), i.e., GS reached 41 L · kg^{-1} after 31 days of incubation, whereas DSS attained this value nearly on the 10th day, stabilizing after that. Regardless of the methane production, the concentration of sCOD varied marginally during the degassing process (Figure 1b). On the other hand, the VS content measured at the end of the incubations (degassed inocula) was lower than in the raw inocula (Table 1), which points to the conversion of particulate material to methane. The $N_{soluble}$ concentration slightly increased over time for both inocula (Figure 1c), which may suggest that nitrogen was being released to the medium, possibly from cell decay. Therefore, the lack of substrate seems to have promoted microbial cell death and their use (or of the metabolites excreted during cell decay) as carbon and energy source by the active microbial communities. This may be the reason for the reduction of the SMA verified at the end of the degassing process (Table 1), and, in particular, for GS, endogenous decay may have led to the slight but constant methane production observed (Figure 1a).

In this work, the sCOD concentration in DM was up to 100-fold higher than that in DSS and GS (i.e., around 472 g · kg^{-1} for DM and 5 to 27 g · kg^{-1} for DSS and GS). This was expected since animal manure is characterized by containing large amounts of residual compounds [34] and leads to a totally different methane production profile. A lag phase of 15 days preceded the onset of methane production, which may be explained by the recalcitrant nature of the residual substrate, making its microbial biodegradation more difficult. Indeed, Yue and co-workers [35] showed that manure contains undigested lignocellulosic biomass plus bedding materials (such as straw), composed mainly of cellulose, hemicellulose and lignin. Polyhydroxy aromatics, resulting from the decomposition of plants, are also generally present in manure [34]. After the lag phase, the methane production rate and solids reduction were higher in comparison with the other two inocula (Figure 1a, Table 1), possibly due to the heterogeneous and complex nature of manure, as well as to its richness in nutrients.

On the other hand, one limitation of the degassing process with DM was the fact that the methane production rate did not stabilize in the studied time frame (~31 days). In accordance with the methane production pattern (Figure 1a), the sCOD of DM started to decrease significantly after 20 days of incubation (Figure 1b), showing that DM needed considerably more time to consume the residual substrate than DSS and GS. Due to the lack of anaerobic digesters of manure nearby, the manure sample used in this work consisted of fresh undigested manure, which explains the high initial concentration of soluble and recalcitrant organic matter. Therefore, for this type of inoculum, pre-incubation is absolutely essential to becoming a suitable inoculum for BMP assays. The initial SAA value determined for DM was very low due to the similar methane production rate recorded in the assays amended with acetate and in the blanks, which resulted from the high sCOD concentration of DM. Therefore, it is not possible to know if the increase in SAA at the end of the degassing process is due to an effective increase in the methanogenic activity or not.

3.2. Effect of Endogenous Methane Production in BMP Tests

The cumulative methane production curves for the assays with each inoculum tested, as well as the volume of methane produced by the blanks, are presented in the Supplementary Materials (Figure S2 and Table S1, respectively).

3.2.1. Digested Sewage Sludge (DSS)

For all the conditions studied, statistically higher ($p < 0.05$) BMP values were reached with raw inocula (DSS) when compared to the assays with the degassed sludge (dDSS) (Table 2). Moreover, when the methane produced in the blanks was not subtracted, the BMP of cellulose largely surpassed the maximum theoretical value (414 L kg^{-1}) for both inocula (Table 2). After discounting the methane produced in the blank assays, BMP values closer to the expected (i.e., 340 to 414 L kg^{-1}, which correspond to 82–100% of the theoretical value) were achieved with dDSS, independently of the Hv Wv^{-1} ratio (Table 2, *BMP_Control* criteria). In particular, BMP values around 97% and 90% of the theoretical maximum were obtained when using blank assays with lower Hv Wv^{-1} ratios (i.e., 0.6 and 2.3, respectively). Prolonging the degassing period (>31 days) to decrease the endogenous methane production does not seem a viable option, considering the occurrence of cell decay and the consequent decrease in microbial activity (as shown by the results presented in Section 3.1). Therefore, the results obtained highlight the practical interest of promoting both the pre-consumption of the residual substrate and discounting the methane produced by the inocula (blank assays) during the BMP tests.

Table 2. BMP values (L kg^{-1}) of crystalline cellulose using digested sewage sludge (DSS) or degassed DSS (dDSS) as inocula, before and after subtracting the methane produced in the different blanks (Hv Wv^{-1} of 0.6, 2.3, 5.0 and 10.1). The corresponding % of the theoretical BMP_{max} is shown between brackets in *BMP_Control* (%). The fulfillment (•) or not (•) of the proposed validation criteria (*CH_4_Blk < 20%*, RDS < 6% and *BMP_Control*) is also shown.

Inoculum	Not Subtracted	After Subtracting Methane from Blanks with Different Hv … Wv^{-1}			
		0.6	**2.3**	**5.0**	**10.1**
DSS	718 ± 7	449 ± 7	437 ± 7	404 ± 6	385 ± 9
CH_4_Blk < 20%	-	•	•	•	•
RSD < 6%	•	•	•	•	•
BMP_Control (%)	• (173 ± 2)	• (108 ± 2)	• (106 ± 2)	• (97 ± 2)	• (93 ± 2)
dDSS	507 ± 3	403 ± 6	371 ± 3	346 ± 6	354 ± 3
CH_4_Blk < 20%	-	•	•	•	•
RSD < 6%	•	•	•	•	•
BMP_Control (%)	• (122 ± 1)	• (97 ± 2)	• (90 ± 1)	• (84 ± 2)	• (86 ± 1)

In all blank assays, the methane generated accounted for more than 20% of the methane produced from the digestion of cellulose (*CH_4_Blk < 20%* criterion). Although failing to accomplish this criterion, discounting the values obtained in the blanks allowed BMP values within the expected theoretical range to be obtained. This is in agreement with the report of the inter-laboratory study [36] that followed the publication of the guidelines from Holliger and co-workers [7], which suggests that the *CH_4_Blk < 20%* criterion should be revised since the production of the blanks was <30% for the majority of tests and there was no correlation between the production of the blanks and the validation of the tests. Based on this suggestion, this criterion was revoked in [18], and the results here presented concur with this decision.

3.2.2. Anaerobic Granular Sludge (GS)

The BMP of crystalline cellulose using GS or dGS as inocula are presented in Table 3, as well as the fulfillment or not of the proposed acceptance criteria. Similar to the previous assays (Section 3.2.1), BMP values higher than the theoretical maximum (414 L kg^{-1})

were obtained when the methane produced in the blanks was not subtracted for both GS and dGS. However, the values obtained after subtracting the blanks were lower than the minimum accepted (i.e., 352 L kg^{-1}) for all the situations studied, representing 72–78% of the theoretical maximum, except for the assay dGS Hv Wv^{-1} 0.6. In addition, criterion *CH_4_Blk < 20%* was not fulfilled in all the cases.

Table 3. BMP values (L kg^{-1}) of crystalline cellulose using anaerobic granular sludge (GS), degassed GS (dGS) and GS degassed for 5 days (dGS-5d) as inoculum, before and after subtracting the methane produced in the different blanks (Hv · Wv^{-1} of 0.6, 2.3, 5.0 and 10.1). The corresponding % of the theoretical BMP_{max} is shown between brackets in *BMP_Control* (%). The fulfillment (•) or not (•) of the proposed validation criteria (*CH_4_Blk < 20%*, *RDS < 6%* and *BMP_Control*) is also shown.

Inoculum	Not Subtracted	After Subtracting Methane from Blanks with Different Hv Wv−1			
		0.6	**2.3**	**5.0**	**10.1**
GS	477 ± 4	301 ± 4	303 ± 4	297 ± 6	306 ± 8
CH_4_Blk < 20%	-	•	•	•	•
RSD < 6%	•	•	•	•	•
BMP_Control (%)	• (115 ± 1)	• (73 ± 1)	• (73 ± 1)	• (72 ± 1)	• (74 ± 2)
dGS	524 ± 1	376 ± 4	321 ± 6	305 ± 5	315 ± 4
CH_4_Blk < 20%	-	•	•	•	•
RSD < 6%	•	•	•	•	•
BMP_Control (%)	• (127 ± 0)	• (91 ± 1)	• (78 ± 2)	• (74 ± 2)	• (76 ± 1)
dGS-5d	454 ± 1	355 ± 6	370 ± 2	370 ± 1	365 ± 3
CH_4_Blk < 20%	-	•	•	•	•
RSD < 6%	•	•	•	•	•
BMP_Control (%)	• (110 ± 0)	• (86 ± 2)	• (89 ± 1)	• (89 ± 0)	• (88 ± 1)

GS is a type of inoculum that does not need long periods of degassing since it is characterized by having a low concentration of residual substrate when compared with other inocula. Moreover, when using GS for BMP assays, the granules can be sieved, removing most of the residual substrate present. Here, GS was subjected to a long period of pre-incubation, which seems to have been detrimental to the anaerobic microbial community (Table 1) and might explain the inaccurate BMP determination (Table 3).

Considering all these results, and also that Angelidaki et al. [3] suggested 2 to 5 days as a typical degassing period for this type of inoculum, a new trial was performed. A new GS sample was degassed for 5 days (dGS-5d) and tested in a BMP assay with crystalline cellulose, using an ISr of 2 g g^{-1} (Table 3). Not subtracting the methane produced in the blanks led again to BMP values surpassing the theoretical value of cellulose, still lower than that of the other two inocula and, consequently, closer to the reference. The time of degassing of the dGS-5d was properly adjusted to ensure only the positive effects of the consumption of the residual substrate, i.e., reducing the possible interferences of the inoculum background methane production in the BMP measurements, while still keeping good microbial activity in the community. Degassing for 30 days was proven to be excessive, being more harmful than beneficial. In the assay with dGS-5d, the methane produced in the blanks reached up to 21% of the total methane produced in the bottles amended with crystalline cellulose. After discounting the blanks, acceptable BMP values were obtained for all the Hv Wv^{-1} studied (i.e., between 86% and 89% of the theoretical maximum), showing that the blanks' headspace volume had no statistically significant effect on the BMP determination and that both degassing and discounting the endogenous methane production (blanks) was advantageous.

The evolution of methane production, sCOD and $N_{soluble}$ was measured in the blanks during the BMP test with dGS-5d (Figure 2). VFA was also analyzed but was never detected. On the first 3 days of incubation, the sCOD concentration decreased from 9 to 6 g kg^{-1} (Figure 2b), and methane was produced at a higher rate (Figure 2a) than after day 3. From

that day on, sCOD and $N_{soluble}$ concentrations increased constantly. Since the solubilization of recalcitrant organic matter does not appear as a possible hypothesis (no VFA was detected and no substrate was present in the medium), cell death and the release of its metabolites to the medium is probably the explanation. Thus, methane production increased linearly for 25 days, until the end of the assay, but at a lower rate (when compared to the rate from the first 3 days), most probably resulting from microbial oxidation of some of their own cellular mass.

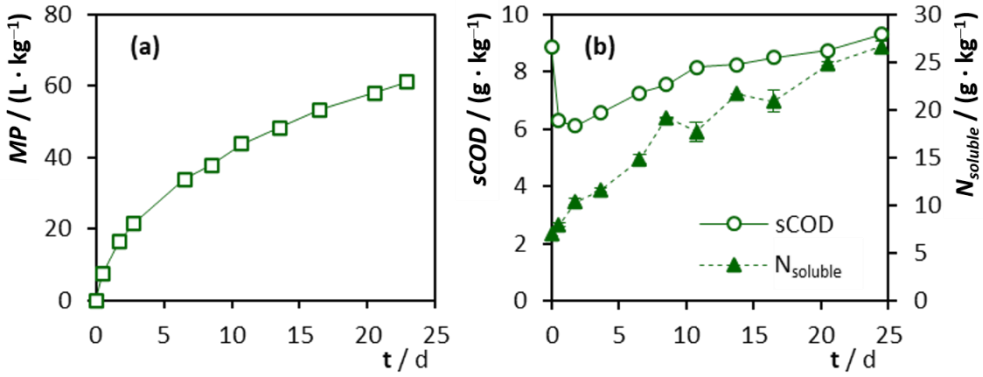

Figure 2. (**a**) Cumulative methane production (MP), (**b**) soluble COD (sCOD) and $N_{soluble}$ concentrations measured in the blank assays during the BMP test with dGS-5d. All parameters are expressed per kg of VS of inoculum.

In summary, two distinct forms of background methane are likely to exist: (i) from the degradation of residual substrate present in the inoculum, constituted by soluble organic matter and also particulate matter (including non-active microbial cells); and (ii) from the endogenous decay of microbial cells. Therefore, in the case of granular sludge, some care should be taken when discounting the final amount of methane produced in the blanks because if the inoculum was subjected to a prolonged degassing period, it will result mainly from endogenous oxidation and probably will not occur in the assays where substrate quantity is not limiting. In this way, the methane produced from the residual substrate in the blanks will be higher than the contribution of the inoculum to the gross methane production measured in the substrate-amended assays, thus underestimating the BMP of the target substrate. Most probably, this was also the case in the assays performed with the GS and dGS, where methane was produced at a faster rate during approximately the first 5 days and after it proceeded at a lower rate (Figure S2c,d).

3.2.3. Dairy Manure (DM)

The results from the BMP assays performed with raw and degassed DM are presented in Table 4. Moreover, the effect of the pre-consumption of inoculum's residual substrate is clearly visible in the determined BMP values. By not subtracting the methane produced in the blanks, the BMP of cellulose largely surpassed the expected, hitting (661 ± 25) and (593 ± 3) L kg^{-1} using DM and dDM, respectively (Table 4). In the assays with DM, by subtracting the methane produced in the blanks with different Hv Wv^{-1} ratios, the BMP of cellulose ranged from 0 to (230 ± 20) L · kg^{-1} (Table 4). As expected, the use of DM as an inoculum caused incoherent results due to the high amount of methane produced by the digestion of manure per se. In fact, the blank assays of DM with a Hv Wv^{-1} of 5.0 and 10.1 produced more methane than the respective digestion of cellulose, yielding a null value for the BMP. Therefore, fresh DM appears to be an unsuitable inoculum for BMP determination since the methane produced from the residual substrate interfered critically in the results, thus reinforcing that degassing is mandatory. dDM achieved BMP values closer to the theoretical, but the values were only within the acceptable range for the lower Hv Wv^{-1} ratio tested (Table 4), i.e., 95% and 83% of the expected BMP was reached using blanks with a Hv Wv^{-1} of 0.6 and 2.3, respectively. The higher Hv Wv^{-1} lead to BMP

values lower than the accepted range. Similar to the previous experiments with the other inocula, the blanks produced more than 20% of the methane produced in the assays with cellulose, regardless of the Hv Wv^{-1} used.

Table 4. BMP values (L kg^{-1}) of crystalline cellulose using fresh dairy manure (DM) and degassed DM (dDM) as inoculum, before and after subtracting the methane produced in the different blanks (Hv Wv^{-1}: 0.6, 2.3, 5.0 and 10.1). The corresponding % of the theoretical BMP_{max} is shown between brackets in *BMP_Control* (%). The fulfillment (•) or not (•) of the proposed validation criteria (*CH_4_Blk* < *20%*, RDS < 6% and *BMP_Control*) is also shown.

Inoculum	Not Subtracted	After Subtracting Methane from Blanks with Different Hv Wv^{-1}			
		0.6	**2.3**	**5.0**	**10.1**
DM	661 ± 25	230 ± 20	115 ± 26	0	0
CH_4_Blk < *20%*	-	•	•	•	•
RSD < *6%*	•	•	•	•	•
BMP_Control (%)	• (160 ± 6)	• (56 ± 6)	• (28 ± 6)	• (0)	• (0)
dDM	593 ± 3	395 ± 15	344 ± 2	294 ± 6	335 ± 16
CH_4_Blk < *20%*	-	•	•	•	•
RSD < *6%*	•	•	•	•	•
BMP_Control (%)	• (143 ± 1)	• (95 ± 7)	• (83 ± 1)	• (71 ± 2)	• (81 ± 7)

The mixture of different inocula is considered an alternative strategy to reduce the amount of residual substrate and minimize its influence on the BMP assays [4,7]. The advantage of this strategy was also verified by Costa and co-workers [37] since a mixture of inocula (suspended sludge and granular sludge) reached higher methane productivities in shrub biodegradation than single inoculum digestions, thus reducing the time of the BMP assay. This acceleration is likely because combined inocula complement a good hydrolytic and acidogenic activity of digested sludge or manure, with a typical excellent methanogenic activity of granular sludge. To evaluate this hypothesis, in this work, a mixture of DM and GS (50:50 %, *w*/*w*) was degassed. Contrary to what was observed during the degassing of DM (Figure 1), methane production started immediately (Figure S3a in Supplementary Materials), as well as sCOD reduction (Figure S3b), shortening the time needed for the consumption of the residual substrate when compared with DM alone. The methane production rate was higher at the beginning of the incubation and decreased thereafter, stabilizing after ~20 days (Figure S3a). In 20 days, the sCOD was reduced by roughly 50%, while for DM alone, sCOD was unchanged during the same period of time (Figure 1b). These results show that the consumption of the residual substrate of DM was accelerated and reinforce that the mixture of different inocula is a promising strategy to be adopted for the preparation of a suitable inoculum for BMP determination using DM.

3.3. Effect on BMP of the Inoculum Source and Blanks' Headspace Volume

The influence of the inoculum source on BMP determination has been studied by several authors, and the differences observed were mainly attributed to the activity and composition of the microbial communities, as well as to the presence or absence of nutrients [38]. In this work, we show that the effect of the inoculum is also related to the amount of residual substrate, which varies for the different types of inoculum. Although some authors have reported no significant differences between the BMP values obtained with non-degassed and degassed inoculum [39,40], this was not the case in our study since we found significantly different BMP measurements for the various inocula tested before and after the pre-incubation period (Figure S4). The duration of the degassing period usually ranges from 5 to 7 days (e.g., [25,29]), although longer (up to 28 days) or shorter (20 h) time periods have also been applied, as reviewed by Ohemeng-Ntiamoah and Datta [38]. In our work, a long degassing period (31 days) had a negative effect on the activity of GS (Table 1) but was necessary for DM due to its high amount of residual substrate (Figure S4).

In general, degassing the inoculum during an appropriate time period brings the BMP values to the acceptable range (i.e., >82% BMP_{max}, Figure S4).

Different BMP values have been reported in the literature for crystalline cellulose. For example, values ranging from 345 to 419 L kg^{-1} were presented in a review of the existing literature data [6], and an average value of (350 ± 29) L kg^{-1} was calculated from data of an international interlaboratory study [41]. The analysis of a large dataset, obtained by more than thirty laboratories from fourteen countries, showed values below 300 L kg^{-1} to above 414 L kg^{-1}, with overall mean BMP values of 346 to 365 L kg^{-1} [13]. Hafner et al. [13] verified that the application of the proposed validation criteria highly improved the reproducibility of the tests, and the cellulose BMP criterion was particularly important. In fact, in our assays, the application of cellulose BMP > 82% BMP_{max} as an acceptance criterion led to the rejection of various situations, namely the assays with dGS (due to the negative effects of the prolonged degassing period) and with DM (due to the interferences that resulted from the excessive amount of residual substrate present in the inoculum). Considering only the results from the assays considered valid, for the different inocula (i.e., DSS, dDSS, dGS-5d and dDM) and Hv Wv^{-1} studied, BMP varied between 344 and 404 L kg^{-1}, with an average value of (372 ± 21) L kg^{-1} (RSD of 5.6%, n = 21), corresponding to 90% of the theoretical maximum. For the different inocula studied, individual BMP values presented less than 6% difference from the average BMP value, with recoveries between 88% and 95% of the theoretical maximum.

For each inoculum, similar amounts of methane were expected in the different blanks (since each bottle received a similar amount of inoculum), and thus the differences observed translate the effect of using different Hv Wv^{-1} ratios (Figure S2 and Table S1). Some differences could be noticed in the BMP values of cellulose due to the different Hv Wv^{-1} tested, but all the Hv Wv^{-1} tested gave BMP values that differed by less than 3% relative to the average BMP and were accurate (recoveries of 87–93% of the theoretical maximum BMP). Therefore, the main point is that care should be taken with the applied practical procedures in order to obtain valid tests (i.e., that fulfill the acceptance criteria), after which the inoculum used and the Hv Wv^{-1} applied will influence the BMP values attained in less than 6%. In the majority of the situations addressed in this work, valid tests were obtained after degassing the inocula and subtracting the background methane from the blanks.

Even so, the analysis of the results obtained for the inocula DSS, dDSS, dGS-5d and dDM show a tendency for the BMP values to decrease with the increase in the Hv Wv^{-1} (except in the case of dGS-5d, for which no significant differences were observed in the BMP values for all the tested Hv Wv^{-1}, possibly due to the fact that this inoculum was degassed, and degassing was performed over an adequate period of time, i.e., 5 days)—see Tables 2–4. For dDSS and dDM, BMP values closer to the theoretical maximum were achieved for the lower Hv Wv^{-1}, in agreement with the proposed hypothesis of less accurate quantification and error propagation in the blanks with higher Hv Wv^{-1}. Only for DSS were BMP values closer to the theoretical obtained for higher Hv Wv^{-1}, and this was probably related to the fact that this inoculum was not degassed and, therefore, higher residual substrate was still present in the blanks.

In this work, the method used to quantify methane (GC with an FID detector) enables the measurement and differentiation of samples in the µmoles range. Even so, in our assays and for the different inocula tested, values as low as 1 µmol were quantified in the 0.5 mL samples collected at the end of the assays in the blanks with higher Hv Wv^{-1}, while for the Hv Wv^{-1} ratio of 0.6, the minimum value achieved at the end of the assay was 12 µmol (Table S2 in Supplementary Materials). When multiplying by the different headspace volumes, these differences were translated into slightly higher amounts of methane (mmol) in the bottles at the end of the assays, ultimately leading to the different final BMP values calculated. It is important to refer that in this work, the bottles were not removed from the temperature-controlled environment during biogas sampling (for GC analysis), and thus the gas samples were collected at a constant temperature. This procedure avoids the errors that may arise from temperature changes, which can influence the headspace

gas composition. In fact, Strömberg et al. [42] verified that temperature was one of the most important parameters to consider in order to avoid gas measurement errors during BMP tests.

4. Conclusions

In the works of Angelidaki et al. [4] and Holliger et al. [7], some recommendations were presented to reduce or avoid potential interferences of the inocula on BMP determinations. However, the results obtained in this work show that even following these recommendations, the BMP of a given substrate may be influenced by the amount of residual substrate, which varies for the different types of inoculum. Additionally, this work shows that the Hv Wv^{-1} ratio of the blanks is also a potential cause of variability in the BMP tests when the accumulated methane is measured by GC.

The use of blanks to subtract the background methane production is mandatory, both for degassed and non-degassed inocula since the theoretical BMP of cellulose was always surpassed when the background methane production was not subtracted. Therefore, it is not adequate to perform only degassing of the inoculum without subtracting the background methane production from the blanks.

Regarding degassing, it should always be performed when the amount of residual substrate is relatively high (e.g., in DM). Otherwise, it may not be possible to distinguish the methane production in the assays from that of the blanks. When the amount of residual substrate in the inoculum is moderate (e.g., in DSS), BMP values within the acceptable range can be found from either degassing or not, as long as the methane from the blanks is discounted. However, in this particular case, degassing first and after discounting the methane from the blanks seems advantageous since it brings the BMP values to the acceptable range. For example, BMP values accounting for 84–97% of the theoretical maximum were achieved with dDSS, independently of the Hv Wv^{-1} used in the blanks. When the amount of residual substrate is low (such as in GS), degassing should be performed for short periods of time. Otherwise, negative effects may arise on the microbial activity of the inocula. In general, degassing the inoculum during an appropriate time period brings the BMP values to the acceptable range (i.e., >82% BMP_{max}).

In the BMP assays, two distinct forms of background methane will possibly occur over time, i.e., methane resulting from the degradation of residual substrate and methane from the endogenous decay of microbial cells. When the residual substrate is low, endogenous decay will also occur in the blanks to a higher extent than in the assays that received the substrate, which may contribute to underestimating the BMP of the substrate.

To guarantee that the BMP of microcrystalline cellulose is higher than 82% of the theoretical and, therefore, that the BMP tests performed with other substrates can be considered valid, adjusting the headspace volume of the blanks to the expected methane production is beneficial. In this way: (i) there is no excess headspace volume that dilutes the methane concentration and that could propagate errors in its quantification; and (ii) reaching high pressure in the headspace is prevented, avoiding the need for depressurization during the test, which promotes errors in the methane quantification method. Considering the assays that fulfilled the *BMP_control* criterion, an average BMP value of (372 ± 21) L kg^{-1} was obtained for cellulose, and the effect of the different inocula or Hv Wv^{-1} was lower than 6%.

The correct selection of the inoculum degassing time and discounting the methane production from blanks with a low Hv Wv^{-1} (adjusted according to the estimated background methane) are essential to reach accurate BMP values and test validation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en15134696/s1, Figure S1: Experimental design of the inocula pre-incubation and BMP assays; Figure S2: Cumulative methane production of cellulose (•) and in the blanks with different Hv Wv^{-1}—0.6 (•), 2.3 (•), 0.5 (•) and 10.1 (•)—in the assays inoculated with (a) DSS, (b) dDSS, (c) GS, (d) dGS, (e) dGS-5d, (f) DM and (g) sDM; Figure S3: Key parameters measured during the degassing process of the mixture of DM and GS (50:50 % *w*/*w*): (a) MP (■), (b) sCOD (•) and $N_{soluble}$ (▲). All parameters are expressed per kg of VS of inoculum added at the

beginning of the assay; Figure S4: BMP values of cellulose obtained for the different inocula after discounting the background methane production from the blank assays with the different Hv Wv^{-1}. DSS (×), dDSS (•), GS (×), dGS-5d (•), DM (×) and dDM (•). The dotted grey lines represent 82% and 100% of the theoretical BMP values (i.e., 340 and 414 L kg^{-1}, respectively); Table S1: Methane production (mL at STP conditions) at the end of the assays in the blanks with different Hv Wv^{-1} (0.6, 2.3, 5.0 and 10.1) for the different inocula studied; Table S2: Methane (µmol) measured in the 0.5 mL samples collected from the bottles with the pressure lock syringe for GC analysis at the end of the assays.

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