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Review

Biochemical Methane Potential (BMP) Assay Method for Anaerobic Digestion Research

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Abstract: Biochemical methane potential (BMP) tests are widely used for characterizing a substrate's influence on the anaerobic digestion process. As of 2018, there continues to be a lack of standardization of units and techniques, which impacts the comparability and validity of BMP results. However, BMP methods continue to evolve, and key aspects are studied to further eliminate systematic errors. This paper aims to update these key aspects with the latest research progress both to introduce the importance of each variable to those new to BMP measurements and to show the complexity required to design an accurate BMP test.

Keywords: anaerobic digestion; biochemical methane potential; energy recovery; sludge treatment

1. Introduction

Anaerobic digestion (AD) has been used for its emphasis on energy conservation and recovery and desire to obtain beneficial use of organic waste [1–3]. Acting through a series of complex microbiological processes, diverse types of bacteria work in an assembly line fashion going through four stages: hydrolysis, acidogenesis, acetogensis and methanogensis [4]. These bacteria are sensitive to environmental conditions, and it is important to balance a range of factors to maximize the chances for achieving optimum design and efficient operation [5,6]. The approach often involves the recognition of the rate-limiting step, which is linked to knowing the characteristics of the organic material being digested. Therefore, the feed characteristics such as toxicity and biodegradability have been found to be major factors for affecting system design and performance [7].

Biochemical methane potential (BMP) tests are a popular technique to determine the methane potential and biodegradability of wastewater and waste biomass [8]. In the test, a substrate is mixed with an anaerobic bacteria culture, normally retrieved from an active digester. The bottles are then stored at a stable temperature of either 35 °C or 55 °C, and constantly mixed for a period of 30–60 days [9,10]. Methane and carbon dioxide are produced during the testing period due to the anaerobic degradation of organic contents of the substrate. The methane generated from the substrate is then measured and the methane potential of the substrate which is expressed as per mass of volatile solids added or chemical oxygen demand (COD) added can be calculated by subtracting the methane volume from a blank. In addition, the substrate may be expressed as in terms of biodegradability by dividing the cumulative methane volume by the theoretical cumulative methane volume, which is obtained from the chemical ratio of 1 g COD = 0.35 mL CH_4 at standard temperature and pressure conditions (STP) [11].

Since the popular methodology of Owen et al. [12] was published, BMP test have been used to characterize a wide variety of substrates and have become important tools for investigating possible pre and post digestion treatment options. As computer models and the complexity of mathematical expressions to describe the anaerobic digestion process improved, the information from

batch experiments have been found to produce reasonable predictions of full-scale behaviour. The BMPs of the substrates to be digested and their specific organic loads could be used to design different components of full-scale anaerobic digestion plants such as the size of the digesters and possibilities of exploiting the produced biogas. For example, Holliger et al. [13] compared the volume of methane predicted by BMP data with the methane volume measured onsite from a full-scale installation over a period of 7 to 9 months. The authors found that the BMP weekly methane production rates were similar and followed the same pattern. In addition, Li et al. [14] found that information obtained from BMP degradation rates could also be used as a practical tool for evaluating process performance in full-scale biogas processes.

Currently, the central issue with BMP tests is the lack of standardized procedures and information required for reporting. Many international and national procedures have been proposed, each using different serum bottles, test inoculum, food to microorganism ratios, nutrients and methane measurement devices. As stated by Pham et al. [15], the most popular methods are Møller et al. [16], Hansen et al. [17], Angelidaki et al., [18] and the Association of German Engineers standard procedure VD1 4630. However, because of a lack of standardized protocol, there have been serious drawbacks impacting the industry user. As the reliability of generated information could be under question due to laboratory specific experimental, operation conditions, and data presentation, limiting the comparability of published results.

In addition, there is the issue of a lack of clear instructions for new operators to start BMP tests. Most BMP methodologies provide general guidelines to accommodate all substrates. As a result, it is difficult for a new operator to design a test with accuracy and confidence due to increased room for variation and misinterpretation. It might be useful to provide methodologies specific to certain groups of substrates. There could be increased confidence in the transferability of the methodology to other labs investigating similar substrates, such as the biodegradability of sludge in the range of 0.5% to 6% solid content. In addition, what is missing from other methodologies is transparency of experimental setups. By being simple and clear through providing an example test setup data such as liquid volumes (which are never shown in other papers), COD mass balance, or the number of bottles used, this would be useful for new labs as it can serve as a model for comparison. Even if labs find there are many areas in need of improvement or obvious sections contributing to inherent inaccuracy, their method could be improved faster because areas in need of development can be more easily pinpointed for their specific lab setup and wastewater sample.

The objective of this paper was to (1) review recent studies that completed experiments to provide insight into key factors such as inoculum, substrate, experimental conditions, operational conditions and data analysis/reporting, as at the time of most protocols, no previous research had been carried out to study the influence of several key factors on anaerobic biodegradability in batch mode, (2) outline an easy to understand BMP serum bottle syringe method for new operators using primary and secondary sludge from a wastewater treatment plant (WWTP) as a case example, and (3) provide the reader with perspective on work investigating future areas of BMP development.

2. Review of BMP Variability Factors

To understand the BMP method, it is important to provide background information discussing each of the required components of a test. The following section goes through the required serum bottle sets, the environmental conditions needed for healthy digestion, the test components quality for wastewater characterization, and techniques used to monitor the progress and health of the anaerobic digestion process during incubation.

2.1. Set-Up of BMP Bottle Test

BMP tests are usually carried out in a volume range depending on the substrate homogeneity. Smaller volumes (125–500 mL) should be used for homogenous substrates, while large volumes (500 to 2000 mL) are more appropriate for heterogenous substrates [9,19]. Smaller bottles may not

ensure realistic operation conditions due to the smaller microbial consortia and reduced volatile fatty acid concentrations (VFA) compared to large scale reactors where higher concentrations of microorganism exist [19]. Pearse et al. [19] recommended that even though larger bottles, due to increased concentrations of microorganism accelerate hydrolysis and VFA build up in the system, they will provide more realistic predictions of gas generation.

BMP tests require a blank, control and substrate. All groups should be performed in triplicates for reproducibility of the tests and statistical analysis. The substrate bottle is filled with inoculum, the substrate, and added nutrients if needed. The blank is filled with the inoculum, a medium or water, but no substrate to provide the background methane generation from the organic material in the inoculum. The control assesses the accuracy of the BMP test using a substrate with a known theoretical methane yield.

The control bottles are filled with inoculum, the control substrate, and added nutrients if needed. To calculate the theoretical reference methane yield value for the selected control substrate, the Buswell formula is commonly used for substrates with known chemical composition (carbon, hydrogen and oxygen) [20,21]. Microcrystalline cellulose, is the most common choice for a control substrate because, as stated by Koch et al. [22] it is relatively easy to calculate the theoretical BMP, its degradation involves all steps in AD, it is cheap, and in high-quality and purity (theoretical methane potential of 415 mL CH_4/g VS at STP) [23]. However, results are rarely 100% accurate when calculating the methane yield of the positive control. There is agreement that during AD, 10% of the substrate is for biomass growth and transformation into heat [23]. This is reflected in the VD1 4630 guideline stating that when cellulose is digested in a BMP test it should produce a biogas yield of at least 80% of its theoretical maximum yield [24]. Similarly, Holliger et al., [9] stated the positive control should achieve at least 85% of the theoretical BMP. Although controls are necessary to provide verification of the accuracy of a BMP method, they are uncommon in BMP papers [25].

2.2. BMP Bottle Environment

It is important to maintain consistent environmental conditions for the microbiology and biochemistry for anaerobic digestion to maximize the chances for achieving optimum performance [5,6]. As stated by Parkin and Owen [7], to ensure efficient digester operation, a balance between the acid-forming and hydrogen-forming bacteria and the methane producers must be maintained. In situations where environmental conditions are nonuniform or unstable the final BMP value can be significantly underestimated. For BMP tests there must be (1) a temperature-controlled environment, (2) proper mixing, and (3) sufficient incubation time for the degradation of biodegradable material.

2.2.1. Temperature

Temperature influences the growth rate and metabolism of micro-organism and the population dynamics in the anaerobic reactor, but also effects factors such as gas transfer rates and settling characteristics of biological sludges. Most anaerobic digesters are operated in either mesophilic (30–38 °C) or thermophilic (50–58 °C) temperature ranges. Thermophilic digestion is faster than mesophilic digestion since the biochemical reaction rates increase with increasing temperature. Additional advantages are increased solids reduction, improved dewatering, and increased destruction of pathogenic organisms [26]. But the use of thermophilic temperatures has a higher energy requirement, a lower quality supernatant with large quantities of dissolved solids, a higher odour potential and much poorer process stability [27]. It is preferred that the temperature of the BMP bottles is the same as the inoculum originating digester. The majority of data in experiments performed at mesophilic temperature, with only some at thermophilic [25]. BMP vessels should be incubated in a temperature-controlled environment with maximum variations of ± 2 °C [9].

2.2.2. Mixing

Mixing influences the distribution of microorganism, nutrients, substrate, alkalinity and the release of gas bubbles trapped in the digester content and prevention of sedimentation of a particulate material and evening out temperature distribution in the digester [7,28–31]. In the case where there is inadequate mixing, inhibition can arise due to the accumulation of toxic metabolic byproductions [29,32]. So far, there remains no optimum mixing pattern for BMP test [32]. Wang et al. [30] studied the influences of no mixing, shaking in a water bath, manually shaking once per day, automated unidirectional and bidirectional mixing for BMP tests. In the experiment, results were found to be dependent on the sludge rheology. When the sludge has a viscous content (12–22 Pa·s at 20 s^{-1}), the highest methane potential and highest maximal daily specific methane production was obtained at the highest mixing intensity [30]. On the other hand, slight stirring or natural movement by the biogas may be enough to avoid inhibition by-productions for sludge with low total solids. The authors further reported that no mixing or manually shaking once per day may be sufficient if the digester content is dilute or easily degraded [30]. However, as a general observation that mixing lacks precision, the mixing condition with BMP tests should try and replicate the basic fluid dynamics of large-scale reactors. Most full-scale reactors are mixed to some extent to reduce solid retention time (SRT) and to release entrained methane.

2.2.3. Incubation Time

Solid retention time (SRT) is regarded as the most important parameter for anaerobic digester design and operation [7]. SRT accurately defines the relationship between the bacterial system and digester operation conditions. Hydrolysis, fermentation and methanogenesis are directly related to the SRT, where an increase or decrease in SRT results in an increase or decrease in the extent of each reaction. As the objective is to determine the maximum volume of methane to be generated from a substrate, the longer the SRT the higher the overall methane production and reduction of biodegradable material. The challenge for the operator has often been selecting an optimal SRT for a substrate that is long enough to ensure efficient conversion of complex organic matter to methane and carbon dioxide, but under time restrictions. In literature reported incubation times range from 30 to over 100 days [25]. These recommendations should only be used as guides. If the daily methane production over three consecutive days is <1% of the cumulated methane production, the test could be finished sooner [33].

2.3. BMP Bottle Contents

2.3.1. Inoculum

Inoculum supplies the microorganism to the anaerobic digestion process, and is one of the most important BMP factors with origin, time of sampling and concentration having the ability to significantly influence results [25,34,35]. Throughout literature there is great variability in the inoculum used in BMP tests, originating from sources such as sewage sludge digesters, agricultural biogas plants and biowaste treatment plants [34,36–38]. Recently, there have been comprehensive studies on the effects of the selection of different inoculums. Most protocol studies state that differently sourced inoculum can lead to different substrate biodegradabilies and flawed data, due to different bacterial population, substrate adaption, and initial microorganism activities [19,36,39,40]. There seems to be a collective conclusion that when selecting inoculum, priority should be the source already adapted to the substrate. The most commonly recommended being the anaerobic digestate from wastewater treatment plants due to the full range of diverse and active microorganisms [19,25].

Part of the standardization of inoculum involves a quality check to indicate whether the operational parameters of the digester are of good quality (see Table 1). The most common recommendation is to pre-incubate the inoculum for 1 to 5 days at 35 °C to degas and reduce the impact of its methane production. Elbeshbishy et al. [40] studied the influence of inoculum pre-incubation and found no significant difference in methane yield or biodegradability compared to non-incubated inoculum, except for higher maximum methane production rates using fresh inoculum at all substrate to inoculum

ratios (SIR). Holliger et al. [9] stated that the decision should be based on whether the inoculum has a low endogenous methane yield (~50 NmL CH_4/g VS). In cases where the total methane production from the blank contributes more than 20% of the total methane production, pre-incubation for exhausting the inoculum might be needed [9].

Table 1. Recommended inoculum conditions for BMP tests.

Parameter	Recommended Range	Units	Reference
Origin Source	Active digester treating municipal wastewater sludge	—	[9,19,35,40,41]
pH	$7 \leq x \leq 8.5$	—	[9]
VFA	<1	g CH_3COOH/L	[9]
NH4	<2.5	g NH_4/L	[9]
Alkalinity	>1.5	g $CaCO_3$/L	[9]
Concentration	15 to 20	g VS/L	[9]
Storage	1 to 5 days at 25 °C	—	[40]
Methane Yield	~ 50	NL CH_4/ g VS	[9]

2.3.2. Substrate

Due to the unpredictable diversity of acceptable substrates and their origins, there are few exact chemical and physical property requirements (see Table 2). Wang [42] recommended that samples should have particle sizes less than 10 mm in any dimension. Substrates should also be analyzed for total solid (TS), volatile solid (VS), volatile fatty acid (VFA), total kjeldahl nitrogen (TKN), ammonium and alkalinity concentrations to design the tests and eliminate potential inhibition problems. In addition, the German standard (VD1 4630) recommended that substrate concentrations should be around 10 g VS/L, when inoculum concentrations are between 1.5 and 2% to achieve inoculum to substrate ratio of 2 [25].

Table 2. Recommended substrate conditions for BMP tests.

Factors	Recommendation	Reference
Particle Size	<10 mm	[18]
Concentration	10 VS g/L	[35]
Compulsory Parameters	TS, VS, pH, VFA, TKN, NH4, ALK, COD	[9]

Wang [42] found the measured methane yield might vary with substrate concentration. In the case of substrates with high concentrations, there is the possibility of overloading the digester, leading to inhibition due to the accumulation of intermediate production. Wang [42] proposed two solutions to minimize the effect of high substrate concentration. One involves lowering the SIR to a more realistic relationship between the sample and the microbial population, as might be found in a full-scale anaerobic digester (hydraulic loading rate/organic loading rate). Option two, requires the dilution of the substrate. Although as shown in Wang [42], the dilution of inoculum or a substrate should be avoided as it might induce underestimations of the methane potential. In Wang [42] experiment the authors used microcrystalline cellulose as the substrate (96.1% VS) and anaerobic inoculum from a mesophilic sewage treatment plant. The BMP of the substrate was then evaluated at increasing VS loads, from 1g VS (2.5 g VS substrate/L) to 6 g VS (15 g VS substrate/L). For each substrate load, three samples were run, one with a dilution using distilled water, a dilution using nutrient/ buffer solution, and no dilution. Results showed that the methane potential (NmL CH_4/g VS) increased with the VS load. The authors noted that if the substrate concentration is too low, there is a possibility of low quantities of gas production due to the low metabolic activity of the microorganism resulting in low methane yield.

2.3.3. Nutrients

Optimal operation of biogas digesters requires balanced concentrations of C:N:P:S (~600:15:5:1), macronutrients (K, Na, Ca and Mg), trace metals (Fe, Zn, Mn, B, Co, Ni, Cu, Mo, Se, Al, W and V) and vitamins to support microbial growth [43]. In BMP tests, any lack can have inhibitory effects [18,44]. Examples of BMP nutrients solutions can be found at Rozzi and Remigi [35], Owen et al. [12], and Angelidaki et al. [18].

In most cases, it is unclear whether BMP tests will have sufficient nutrients available from the sludge and substrate or if additional supplements are necessary. In some cases, nutrients supplementation can be avoided when the seed is suspected of having enough nutrients and the seed volume can prevent reactor acidification [33]. Wang et al. [42] studied the impact of a BMP set using no dilution, distilled water and nutrient/buffer solution on methane yield and degradation rate. Positive effects on degradation rate was found when nutrients were added, but regarding the final methane yield calculation there were minor differences in comparison to the strong effects of the choice of substrate concentration. In the situation where, digested sewage sludge is the inoculum, nutrient supplementation could be exempted. As stated by Shelton and Tiedje [45], digestate is likely to have all mineral and metal nutrients in amply supply (except for potassium, ammonium and cobalt), and the addition of excessive nutrients could be inhibitory.

2.4. BMP Testing Monitoring

2.4.1. Biogas Monitoring

As the organic material in the substrate is degraded through a series of complex microbiological processes, biogas is continually produced during incubation until there is no biodegradable material left. Since biogas production is the key factor to determine the methane potential and biodegradability of a substrate, it is important for the BMP method to both collect the biogas without significant losses or error and apply correction factors to convert the observed methane potential to standard temperature and pressure conditions for standardized results [46]. Techniques for measuring the rate and volume of biogas produced from anaerobic biodegradability assays include: lubricated syringes, volume displacement devices, pressure manometers or transducers, manometer assisted syringes, or low flow pressure.

(1) Syringe Method

In the case of syringe method, a glass syringe is inverted straight into the lid of the reactor. The overpressure inside the reactor pushes the piston until there is balanced in the pressure buildup to atmospheric pressure [8]. The volume of biogas can be read off the syringe. The gas can be injected back into the bottle or wasted. An added advantage of venting the biogas produced is that headspace pressures and the carbon dioxide solubility in the bioreactor vessel can be kept to a minimum.

However, this method, due to its manual operation has potential areas for human error. In most cases, the incubated bottles are removed from the temperature-controlled environment during the measurement of gas. This change of temperature can easily affect the equilibrium between the gas and liquid phase which can result in the change in headspace gas concentration and microbiology of anaerobic digestion [47].

(2) Liquid Displacement

In the volumetric methods, the produced biogas can move into an external collection system that measures the volume. In liquid displacement, a vessel is filled with a barrier solution and inverted in a reservoir. As biogas is produced, it passes through the liquid vessel and displaces an equivalent liquid volume. A prevent issue with this method is the dissolution of CO_2 into the barrier solution. Different setups use different liquids such as tap water, oil, acidified water and carbonated water, but each need to use different correction factors [47]. Gas solubility errors can be eliminated by collecting

gas in a gas bag and measuring the gas volume with liquid column meters. Zaman [33] recommended using a suitable barrier solution to avoid CO_2 diffusion, such as highly acidic or saline. The use of displacement gasometers requires that measurements taken directly from the gas column (liquid levels, pressure) are used to calculate gas volumes. As well as adjusting to STP, it is also necessary to consider the vapour content and correct for any hydrostatic pressure on the gas [15].

Pham et al. [15] compared the intermittent measurements with syringe (1000 mL), intermittent measurements with liquid replacement system (LRS), and continuous measurements with liquid replacement (CLRS). All three techniques were used for the VD1 batch fermentation method of pig manure, cow manure, cellulose and inoculum samples. In the case of cellulose, CLRS, LRS, and the syringe determined the methane yield to be 537.79 ± 9.10 NL/kg VS, 571.36 ± 10.24 NL/kg VS, and 583.76 ± 5.94 NL/kg VS. The results showed that the liquid replacement system had a tendency for higher gas volume measurements than the syringe and CLRS methods. The reason could be that the syringe plunger was not withdrawn far enough to get the total production in each test and left a higher pressure in the headspace, or that in the case of the CLRS method there were small leaks in the setup, as the biogas is contained not only in the digester but also through the whole water replacement system. However, the difference in the gas volumes obtained using three different measurement techniques were much less than the differences caused by different fermentation procedures and gas measurement techniques [15]. Therefore, the authors concluded that the differences between the tested methods were not significant.

(3) Manometric

Manometric methods using the pressure transducer require the pressure to build up inside the reactor. This method is easier to perform than the liquid displacement but requires more effort in the test setup, and depending on the gas to liquid ratio, accuracy can be sensitive to the gas non-ideal behaviour, change in gas space volume during the test, dissolution of methane and CO_2 in the liquid [35]. Zaman [33] stated that the main drawback of the manometric approach is that variation in the pressure of the headspace gases alters the quantity dissolved in the liquid phase, especially carbon dioxide.

Manometric and volumetric biogas measurement techniques were compared by Raposo et al. [34] in an inter-laboratory study on methane produced by cellulose. In the inter-laboratory study 19 laboratories participated. Volumetric methods were used most (63%), followed by manometric methods (26.3%) and by GC methods (10.5%). Laboratories using manometric method reported lower methane yield for cellulose than those using volumetric BMP methods [24]. Similar results were found Himanshu et al. [24] in a review of Logan et al. [48] who reported a lower biogas yield with a manometric method compared to a variation of the volumetric method [24]. Although the measurement of the biogas production using a pressure transducer as the detector is easier and more reliable than the liquid displacement, errors related to CO_2 solubility in the bioreactor liquid can still lead to underestimation of biogas production if not accounted for [49].

(4) Biogas Composition Monitoring

Methane production, as a process performance indicator is one of the most sensitive since it is directly related to organic matter destruction. Typical values of percent methane for digesters operating on municipal wastewater sludges are 60–75%. During system imbalance, methane production and total gas production will decrease, while the percent CO_2 will increase [7]. Gas chromatography (GC) is often used for its high resolution, high sensitivity and quantitative results, to measure the content of methane and carbon dioxide in a biogas sample [11]. However, as found by Parajuli [47], varying temperatures and water vapour content in the biogas sample can cause measurement errors. Parajuli [47] studied three potential temperature difference between the sampled biogas and standard calibration gas. The samples were humidified in comparison to dry standard. In the temperature and dry and humidified biogas measurement tests, a synthetic gas of 50% CH_4 and CO_2 were measured

at temperature 5 °C, 25 °C, 55 °C and 70 °C using a calibration standard at 23 °C. At 35 °C, the concentration of 50% CO_2 and CH_4 was 52.1% for dry gas and 53.4%, respectively. At 55 °C the results were 56.9% and 58.8%. Ideally, the best solution would be to use a vapour saturated standard, but this would be laborious and time consuming. The author found that rapid injection of samples without delay and the use of insulated syringe would give more precise results.

An alternative to the GC to measure CH_4 in biogas can be determined by the liquid replacement method [11,15]. Pham et al., [15] compared the measured CH_4 concentration using liquid replacement method (LRM) and a GC. The LRM generally had higher CH_4 concentrations (68%) in comparison to the GC (64.94%). The authors stated that due to the very low differences, the LRM could be a viable alternative for measuring biogas content for laboratories with limited access to expensive equipment.

2.4.2. Liquid Monitoring

Measurement of the physical and chemical composition of the digested liquid can be carried out regularly to monitor the digester environment and performance. Imbalanced digestion can be triggered by changes in organic or hydraulic loading, changes in organic feed characteristics, changes in temperature, or introduction of toxic substances. During imbalanced digestion, typically volatile acid concentrations increase while bicarbonate alkalinity, pH, gas production, percent methane, and the destruction of organic matter all decrease. Careful monitoring of these variables should allow operations personnel to observe the onset of stress and take appropriate remedial measures to prevent system failure.

To monitor the changes in concentrations of these liquid phase parameters, it is necessary to set up a certain number of "sacrifice bottles" in BMP tests. These extra bottles (identical to the blank and substrate sets) can be sacrificed by being periodically opened throughout the experiment period to provide samples for the liquid phase parameter measurement. For instance, due to the nature of the methane yield first order curve, the start-up period often slows/ends after 10–12 days before plateauing. Therefore, the operator might place extra bottles could be taken out at the initial start, after 5, 10, 15, and 30 days for analysis. Monitoring liquid phase parameters (TCOD, sCOD, VFA, total NH_3 nitrogen, TKN, TN, TP, PO_4-P, pH, alkalinity, TS, VS, TSS, VSS, EPS components of proteins, carbohydrates, and humic acids, etc.) could allow the operator to assess the performance of the anaerobic digestion process, determine reaction stability, and identify potential inhibitory factors [7].

(1) pH

A narrow operating range of 6.5–7.6 is often recommended, since pH influences the microorganism enzymes and can change their configurations and influence the kinetics of the reactions [7,8]. A low pH can bring about an accumulation of VFA, which inhibits digestion [7,19]. This can occur when a substrate with sufficient inhibiting substances (NH_3, H_2S and heavy metals) is added into the serum bottle, or when bottles are exposed to transient temperature changes [8,20]. As a result, unstable operations can develop as the VFA production rate exceeds the methanogenic VFA utilization rate. As the pH lowers, the VFA utilization kinetics and methanogenic activity decreases: advancing VFA accumulation, inhibiting methane production and resulting in a process failure [8]. Both methane and carbon dioxide content can be used as indicators of an upset. Typically, the methane content of biogas is in the range from 60 to 75% with carbon dioxide comprising the remainder. Large decreases outside this range could indicate a failing test. A high pH, on the other hand, can be inhibiting due to concentrations of free ammonia (FA) and ammonium ions. FA has been suggested to play a major role in inhibition because it can freely pass through the membrane of the microorganisms and diffuse into the cell, leading to proton imbalance and potassium deficiency [31,50]. Ammonia concentration (NH_3-N) of less than 200 mg/L are beneficial for the AD process as it is an essential nutrient [31]. According to Parkin and Owen [7], researchers suggest that FA concentrations above 100 mg/L can cause toxicity.

Normally, an alkalinity in the range of 2000 to 3500 mg/L as $CaCO_3$ is needed to maintain the pH at neutral. In the BMP tests, the production of VFA will reduce the alkalinity while the production of NH_3 from protein and amino acid deamination will increase the solution alkalinity. For the materials that have a high protein contents, there will be less likely to see a significant drop in pH in BMP.

(2) Monitoring Solid Concentration Reductions

Sacrifice bottles can also provide insight into the kinetics of the reaction process by observing the reduction in solid concentrations (see Figure 1). The destruction of organic matter is the primary objective of anaerobic digestion. Therefore, COD and VS must be measured to determine the overall process efficiency. Monitoring physical properties of wastewater is important to assess the reusability of the wastewater and determine the most suitable type of process for its treatment. As shown in Figure 1, TS, VS, TSS and VSS measured over 30 days for both blank and test bottles could provide insight in to which parameter has the greatest reduction.

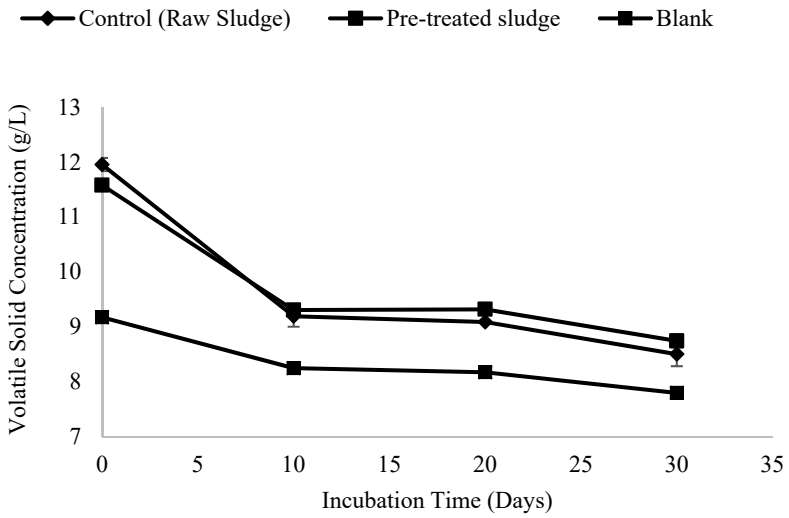

Figure 1. Example of solids reduction measurements during 30-day BMP test.

But as an indicator of imbalanced digestion, organic matter destruction is not a sensitive measurement of process imbalance. It will only confirm what trends VA, pH, TALK and methane production have already shown. Frequent monitoring of influent COD and VS levels may help determine if system imbalance was caused by increased organic loading (reduction in effective SRT) and may help to predict and minimize detrimental effects if the monitoring is frequent enough [7].

(3) Mass Balance

COD mass balances can assist in validating results and making them comparable [51]. COD mass balances can be carried out because COD is not destroyed but re-disturbed in anaerobic digestion. Theoretically, the COD in the influent is equal to the COD leaving the system, which occurs through effluent, methane generation or incorporated into new bacterial mass [52].

$$COD_{influent} = COD_{effluent} + COD_{gas} + COD_{sludge} \quad (1)$$

The methane COD can be calculated using the empirical relationship, where 1 kg COD can be converted into 0.35 m^3 CH_4, and the COD difference between the COD influent and COD effluent [52] (see Equations (1) and (2)). However, the COD mass balances of a reactor will not be 100%. If the liquid COD measurements are accurate, the gap between could provide insight into the amount of newly grown and entrapped biomass [51]. But to complete a perfect COD mass balance is difficult in

accounting for fates of COD in the anaerobic digestion process and potential errors in measuring the COD in the anaerobic liquid.

$$V\left(\frac{m^3}{d}\right) = 0.35\ \frac{m^3CH4}{kg\ COD} \times (CODi - CODe)\left(\frac{kg}{m^3}\right) \times Q \quad (2)$$

There are various fractions in the anaerobic digestion process contributing to a gap in the COD balance. Lier et al. [52] reported the relative importance of the indicated COD fraction in influent, effluent, sludge, and biogas in terms of soluble organic/inorganic, suspended organic/inorganic, absorbed, entrapped CH_4, H_2, H_2S, N_2 and newly grown biomass. The authors discussed two frequently cited causes for the COD gap. One occurs when there is a "loss of electrons" to oxidise anions like SO_4^{2-} and NO_3^-, and the other is when COD is entrapped or accumulates in the sludge bed. The latter situation occurs when the wastewater being treated had a high fat or long-chain fatty acid (LCFA) content. In these situations, the combination of high measured COD removal efficiency but low methane production rates could lead to large gaps in the COD balance, indicating long-term operational problems [52].

Moreover, there remains a question of the accuracy of COD measurements for solid and liquid samples with high suspended solid content in anaerobic research. Raposo et al. [34] stated, directly measuring COD is thought to produce erroneous results. Angelidaki and Sanders [11] listed possible reasons that might cause problems during COD measurements as (1) volatile straight-chain aliphatic compounds are not oxidized to any appreciable degree, (2) aromatic carbohydrates, and some aromatic heterocyclic compounds are not oxidized, (3) NO_2-2 exerts a COD of 1.1 mg/mg NO_2-N, and (4) reduced inorganic compounds such as ferrous iron, sulphide, manganese are oxidized quantitatively under the species [11]. In 2008, the first Proficiency Test (PT) of COD was completed with 26 labs from 16 countries to measure the COD of two solid samples and two high concentrated suspended solid samples [53]. All participants used potassium dichromate as the oxidant reagent but with different experimental procedures. Out of the total participants reporting data (26 labs), 36% of results were satisfactory, 9% doubtful, and 5% unacceptable. Only two labs (8% of participants) reported the four samples adequately. The short-term conclusion was that solid samples and liquid samples with high solid concentration could not be analyzed accurately. A second PT was carried out in 2009. In comparison with the previous results, the overall performance improved by 30%, respectively [54]. Raposo et al. [54] interpreted it as a sign of general improvement, and possible to accurately measure the COD of difficult samples with acceptable quality. Despite the sensitivity of obtaining perfect mass balance results, COD mass balances should be developed. They can still be useful trouble shooting tools for new laboratories starting conventional BMP tests.

2.5. Data Quality and Reporting

2.5.1. Complexity of Methane Correction

Standardized accumulated methane volume measurements are important for reliable and comparable BMP and rate constant values. But corrections of methane volumes to standard conditions are often poorly communicated in published experiments. This often involves uncertainty due to the missing information about emerging factors such as temperature, pressure, water vapour and headspace composition. According to Strömberg et al. [10], most scientific papers in the field of anaerobic digestion simply quote gas production volumes without mentioning any corrections applied to standard conditions. Strömberg et al. [10] completed a short literature study on gas normalisation of 23 papers (exclusively on the digestion of cattle manure). One out of the 23 correctly accounted for temperature, pressure and water vapour. Eight reported a correction for temperature and pressure but not water vapour, and seven were missing correction information.

The main confusion for researchers converting methane volumes could be focused on two factors: (1) confusion about which standard reference conditions to adopt, and (2) confusion about which

correction equation to use depending on the type of biogas monitoring technique. As stated by Parajuli [47], there is an issue when there are different standard reference conditions. For example, the National Institute of Standards and Technology uses 101.325 kPA and 20 °C, while the International Union of Pure and Applied Chemistry uses 100 kPA and 0 °C [47]. But also selecting from the variety of correction equations reported in literature for syringe (Equation (3)), liquid displacement (Equation (4)) and manometer (Equation (5)). The major difference between each technique being the decision to adjust for water vapour, include overestimate correction factors, or which order in which equation to adjust for temperature and pressure. Until there is clarity about a single method, or clarity of conversion for each of the three methods, there may always be some question about the validity of methane corrections. It is estimated that in the future, this area will be central for the standardized method.

Syringe Method [55]:

$$V_{CH_4,t} = V_{biogas,t} \times \frac{\%CH_4, t}{100} + Vh \times \frac{\%CH_4, t - \%CH_4, t-1}{100} \quad (3)$$

Liquid Displacement Method [10]:

$$V_{acc,i} = V_{acc,i} + (VM - VOE, i) \times \left(1 - \frac{Pvap, i}{Pgas, i}\right) \times \left(\frac{Pgas, i}{PSTP}\right) \times \left(\frac{TSTP}{Tgas, i}\right) \quad (4)$$

Manometer Method [56]:

$$V_{CH_4,t} = \left(V_{headspace} \times \frac{TSTP}{T} + V_{biogas,STP}\right) \times \left(\frac{\%CH4\ dry,current}{100}\right) - \left(V_{headspace} \times \frac{Tstp}{T}\right) \times \left(\frac{\%CH4dry,previous}{100}\right) \quad (5)$$

2.5.2. Methane Curve Interpretation

Understanding the meaning of the methane yield curve could provide the operator insight into the rate limiting step of the test material during anaerobic digestion (see Figure 2). As stated by Remigi and Buckley [8], there are four possible interpretations of a methane yield curve of the test material. Curve 1, the test material is readily biodegradable. Biogas and methane are immediately produced, and the methane yield curve quickly levels off. Curve 2, the test material is biodegradable after a lag phase. A lag phase could indicate hydrolysis as the rate limiting step in the anaerobic digestion process. Curve 3, the test material is inhibitory in the initial phase of incubation. In this case, the test material contains toxic substances that are inhibiting the microorganisms, causing the test material methane production to be lower than the blank. For this reason, when the methane is subtracted from the blank, the methane yield becomes negative. Curve 4, the test material is inhibitory throughout the entire period of incubation. The test material contains toxic substances inhibiting methane bacteria and hydrogen producing and consuming bacteria. No methane is produced in comparison to the blank which is slowly producing methane. Therefore, as the test continues and the substrate bottles continue to no produced biogas, the methane yield becomes increasingly negative.

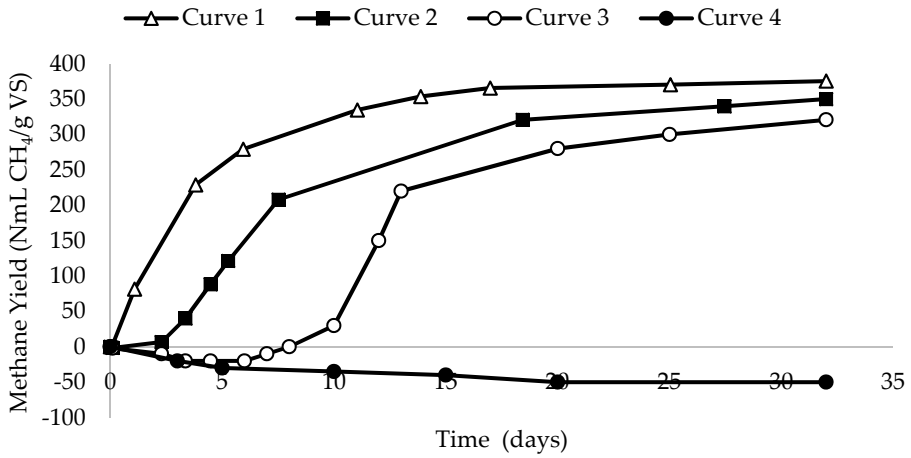

Figure 2. Example of different methane yield curves for four different test materials.

2.5.3. Kinetics

BMP kinetic rate constant (k) provides useful information of degradation kinetics of materials to achieve optimal design and operation of anaerobic digesters. But finding the correct value is difficult to achieve, as it is more sensitive to the experimental conditions than the methane yield [57,58]. In published literature, many kinetic models have been used to describe the methane production of BMP tests (first order rate model, Monod type model, modified Gompertz model, a combination of two first order rate models, Chen and Hashimoto model) (see Equations (6)–(10)).

First order rate model:

$$BMP(t) = BMP_{\infty} \times (1 - \exp(-k \times t))) \quad (6)$$

Monod Type Model:

$$BMP(t) = \frac{BMP_{max} k \times t}{k \times t + 1} \quad (7)$$

Modified Gompertz model:

$$BMP(t) = BMP_{\infty} \times \exp\left\{-\exp\left[\frac{u_m e}{A}(\lambda - t) + 1\right]\right\} \quad (8)$$

A combination of two first order rate models:

$$BMP(t) = BMP_{\infty}(1 - X \times \exp(-k_1 \times t) - (1 - X) \times \exp(-k_2 \times t)) \quad (9)$$

Chen and Hashimoto Model:

$$BMP(t) = BMP_{\infty}\left(\left(1 - \frac{k}{HRT \times \mu m + k - 1}\right)\right. \quad (10)$$

Determination of Kinetic Constant

Currently, there is no standardized model to apply to all BMP results. The variability of the selection of the model is based on the substrates used. There are some common models that are more accurate and applicable than others [59]. Kafle and Chen [60] compared the first order model, to the modified Gompertz model and Chen and Hashimoto model. The first order model showed better fit than the modified Gompertz, but when a lag phase was reported the modified Gompertz model better predicted the BMP compared to the first order. Strömberg et al. [61] evaluated of six different kinetic models (first order rate model, a first order rate model with variable order of time dependency, a combination of two first order rate models, a Monod type model, a quadratic Monod type model and

a modified Gompertz model) in predicting the final BMP and test time. The Monod type, quadratic model and first order had positive effects on BMP predictions. While the first order, two combined first order and the modified Gompertz had negative impacts. Chao et al. [62] compared the first order model, modified first-order model and the Gompertz model to fit the BMP curve of wheat straw, separated stem. The modified first-order model had the highest simulation precision, while the first order model had the lowest precision. The maximum BMP value simulated by the Gompertz was the closest among the three models. As a generalization, the first order model is used for fast and abruptly stopping degradation, Monod model better describes the slowly declining gas production at the end of the process, the combination of two first order equations are used when a substrate has two separate degradation profiles, the modified Gompertz equation can be used when a lag phase is present.

It is difficult to compare kinetic constants due to the complex nature of each individual experimental setup (particle size, origin of inoculum, mixing rate and temperature). One of the most studied aspects of influence being the SIR. Multiple studies have shown the variability of kinetic constants of BMP methane production with the substrate to inoculum ratio (Hashimoto [63] with ball-milled straw, Raposo et al. [64] with sunflower oil cake and Moset et al. [65] for maize.). In most cases, high hydrolysis rates were reached in anaerobic biodegradability tests with a low SIR, showing a degree of dependence of hydrolysis to inoculum concentration and activity. As stated above, an experiment that choses one SIR from recommended may not be the optimal ratio for a specific substrate, possibly underestimating the value.

2.5.4. Data Rejection and Data Reporting

Holliger et al. [9] stressed the often-unaddressed area of quality control, stating that data must pass some quality criteria for use such as than the test results must be rejected:

1. If the relative standard deviation (RSD) of the blank or the positive control is >5%, even after applying a statistical test to eliminate a single outlier;
2. If the RSD of a homogenous substrate is >5%, even after applying a statistical test to eliminate a single outlier;
3. BMP of the positive control <85% or >100% of theoretical BMP.

The analysis and presentation of BMP data are one area often left partially addressed in most standard procedures, specifically the type of equipment and applied experiment set-up, which many times are self-developed and specific for each laboratory. Details that should be accounted for in the final report were combined from Angelidaki et al. [18] and Holliger et al. [9]. The following details should be presented in the final report:

1. Inoculum and substrate physiochemical characteristics;
2. Test Conditions and setup;
3. Graphs of gross methane production of the substrate batches;
4. Positive controls and blanks.

2.6. Summary

As of 2018, there continues to be a lack of standardization/universal BMP testing procedure, limiting the comparability of results. However, BMP methods continue to evolve, and key aspects studied to further the elimination of systematic errors. In this paper, key aspects of proposed BMP methods were reviewed and summarized with the latest research progress to inform a simplified serum bottle method. Updating these recommendations may increase the probability of obtaining validated and reproducible BMP.

3. BMP Serum Bottle Syringe Method for Wastewater Sludge Anaerobic Digestion Studies

The BMP serum bottle method was outlined in the following section to determine the key steps and parameters of the BMP test to characterize methane production potential and biodegradability of WWTP primary and secondary sludge (see Figure 3). Serum bottle syringe method was chosen for its flexibility, quick set up and ease of use. The objective was to structure the following sections for a new operator to increase the ease of starting new tests to provide insight into their anaerobic digester system. This BMP serum bottle method procedure has four main components: (1) test preparation, (2) test start up and operation, (3) data analysis, (4) data presentation.

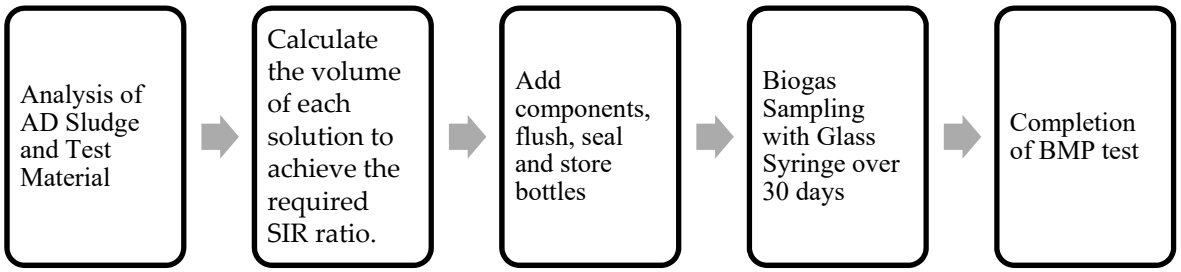

Figure 3. Flow diagram for BMP procedure.

3.1. Materials

The materials required for the BMP serum bottle syringe method include:

1. Batch anaerobic digester containers: 125 mL glass serum bottle (Wheaton: Millville, New Jersey, USA) (total volume 160 mL);
2. Temperature controlled environment (incubator): New Brunswick Scientific C25 Incubator Shaker Classic (New Brunswick Scientific: Edison, NJ, USA);
3. Flush gas: Pure nitrogen;
4. Biogas Production Measurement Device: 10–50 mL glass syringes (Cadence Science Inc.: Cranston, RI, USA);
5. Gas Composition Analysis: Agilent 6890 GC system (Agilent Technologies Inc.: Wilmington, DE, USA) with TCD. Argon was used as the carrier, with an inlet temperature of 200 °C;
6. Characterization of inoculum and substrates: Apparatus for the determination of COD, solids, alkalinity, and VFA.

3.2. Test Preparation

Anaerobic digester, primary and secondary sludge were collected from the Guelph, Wastewater Treatment Plant (GWWTP). The GWWTP is located at Guelph, Ontario, Canada and provides treatment of domestic, commercial, institutional and industrial wastewater collected from the community of the Guelph/Eramosa [wastewater treatment plant annual report]. The Guelph WWTP process consists of preliminary screening and grit removal, primary sedimentation, extended aeration activated sludge treatment, secondary clarifications, rotating biological contactors (RBC) and sand filtration tertiary treatment, and chlorine disinfection. The typical wastewater daily average flow treated by the Guelph WWTP is 50.02 ± 15.6 ML, which contained a cBOD5 of 193.4 ± 15.6, TSS of 257.2 ± 27.1, total phosphorus of 5.14 ± 0.38, TKN of 38.5 ± 2.9, and NH_3-N of 22.3 ± 1.6 mg/L according to the annual average values from 2011 to 2015, and the recorded removal efficiencies for cBOD5, TSS, TP, TKN, and NH_3-N are around 98.8%, 99.2%, 97.0%, 95.9%, and 97.9%, respectively. The raw sludge produced in the GWWTP is thickened in the primary clarifiers and further thickened to a sludge of 4.3% solid content by a rotary drum thickener and send to the anaerobic digesters. The WWTP plant generated 27,529 m^3 of thicken sludge per year to the anaerobic digesters which were operated at a SRT around 15 days.

Total solids (TS), volatile solids (VS), mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) were determined by standard methods (Method 2540-1997, and EPA Method 160.4). Chemical oxygen demand (COD) and volatile fatty acids (VFA) of tested samples were determined using Hach test vials (Hach, London, ON, Canada). Raw or pretreated secondary sludge was centrifuged at 10,000 rpm, 4 °C for 15 min, and the supernatant was filtered through a 0.45 um syringe filter, and the pH of filtered sample was determined by TitraLab®870 titration workstation (Radiometer Analytical SAS, Lyon, France).

Table 3 summarises the main characteristics of AD sludge of the Guelph WWTP for different sampling times. The AD sludge showed a stable TS content of 19.6 ± 0.3 g/L over the sampling period, which was very close to the annual average TS 19.5 g/L over the period of 2011 to 2015. The VS/TS and VSS/TSS ratio of the AD sludge were determined to be 0.63 ± 0.14 and 0.70 ± 0.12, respectively. The relative stable TS and VS/TS ratio with the AD sludge suggests that the AD digesters of the WWTP can provide biological consistent inoculum for the sludge BMP tests. The inoculum AD sludge was stored in 2 L sealed plastic bottles with the headspace flushed with 100% nitrogen, and kept in the incubator at 35 °C for 1 to 5 days to degas and reduce the impact of its methane production [18,34].

Table 3. Inoculum characteristics.

Parameter	Units	17 May 2016	2 June 2016	7 June 2016	24 June 2016
TCOD	mg/L	17,160 ± 350	18,460 ± 221	17,060 ± 222	19,000 ± 240
SCOD	mg/L	250 ± 9	525 ± 0.33	546 ± 34	647 ± 20
TS	g/L	19.08 ± 0.02	19.28 ± 0.16	19.74 ± 0.10	19.74 ± 0.25
VS	g/L	16.05 ± 0.07	11.07 ± 0.16	10.92 ± 0.03	10.92 ± 0.12
TSS	g/L	11.05 ± 0.39	18.14 ± 0.28	18.40 ± 0.19	18.40 ± 0.48
VSS	g/L	9.71 ± 0.28	11.06 ± 0.18	12.17 ± 0.04	12.17 ± 0.38
ALK	mg$CaCO_3$/L	4825 ± 19	5867 ± 95	5187 ± 173	4772 ± 160
pH	—	7.7 ± 0.1	7.6 ± 0.1	7.4 ± 0.1	7.6 ± 0.1

Primary and secondary sludge were passed through a 4.75 mm sieve to remove any large particles and analyzed to determine total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD) according Standard method (Method 2540-1997, and EPA Method 160.4). The characteristic parameters of the primary and secondary sludge are shown in Table 4. Compared to the secondary sludge, the primary sludge had a much higher TCOD, SCOD, TS, and VS contents and VS/TS ratio. The alkalinity of primary sludge was also significantly higher than the secondary sludge.

Table 4. Substrate characteristics.

Parameter	Units	Primary Sludge	Secondary Sludge
TCOD	mg/L	47,055 ± 2991	10,670 ± 254
SCOD	mg/L	1945 ± 83	58 ± 6
TS	g/L	36.02 ± 0.22	10.36 ± 0.25
VS	g/L	26.09 ± 0.24	6.63 ± 0.12
TSS	g/L	33.57 ± 0.24	9.32 ± 0.48
VSS	g/L	25.03 ± 0.17	6.55 ± 0.38
ALK	mg$CaCO_3$/L	2333 ± 175	83.6 ± 0
pH	—	6.5	7.36

3.3. Design Calculations

To make sure the BMP is carried out in conditions that are not limiting or inhibiting of the anaerobic digestion process, each BMP test will be designed differently depending on the inoculum and substrate concentrations. This involves adjusting both the inoculum and test material volumes until the (1) estimated gas production, (2) substrate to inoculum ratio, (3) reactor VFA/Alkalinity Ratio,

and (4) headspace to total solution volume, are all balanced within their recommended parameter ranges (see Table 5).

3.3.1. Substrate to Inoculum Ratio (SIR)

In order to find the maximum methane potential and methane production rate, the right balance between the substrate and microorganisms are needed [66–69]. As stated by Raposo et al. [25] theoretically, the methane yield should be independent of the SIR, and the SIR only affects the kinetics of the methane production. However experimental data shows that SIR can have an influence on both, due to the strong evidence that the ratio directly affects the growth patterns of microorganisms [25,70,71]. As a baseline, Owen et al. [12] first proposed, that 1 g VS substrate/g VS should be used [12,34,41]. The German standard, VDI 4630 recommended a SIR of less than 0.5 [31]. Although this provides a useful guideline for the selection of SIR, different substrates may react differently. As stated by Elbeshbishy et al. [40], there is a wide range of optimum SIR depending on the substrate and inoculum.

The authors investigated the influence of SIR ratios on the methane yields and kinetic constants of the primary and secondary sludge by varying the SIR from 0.1, 0.5, 1, 1.5, and 3 g substrate COD/g inoculum VS. In these tests, the total working volumes were set to 55 mL and 60 mL for the primary and secondary sludge BMP tests, respectively, while the volumes of the substrate and inoculum were varied to achieve the desired SIRs. For the primary sludge tests the substrate/inoculum volumes were 2 mL/53 mL, 7 mL/48 mL, 12 mL/43 mL,15 mL/40 mL, 23 mL/32 mL and for secondary sludge 5 mL/55 mL, 20 mL/40 mL, 30 mL/30 mL, 25 mL/35 mL, 45 mL/15 mL. The blanks were used for each condition by replacing the substrate with the same volume of deionized (DI) water. Triplicates of BMP bottles were used for every testing condition. Figure 4 depicts the methane production increased with increasing SIR at a linear fashion. Based on these results, the differences in methane production were due to the increase of organic matter added into the serum bottles.

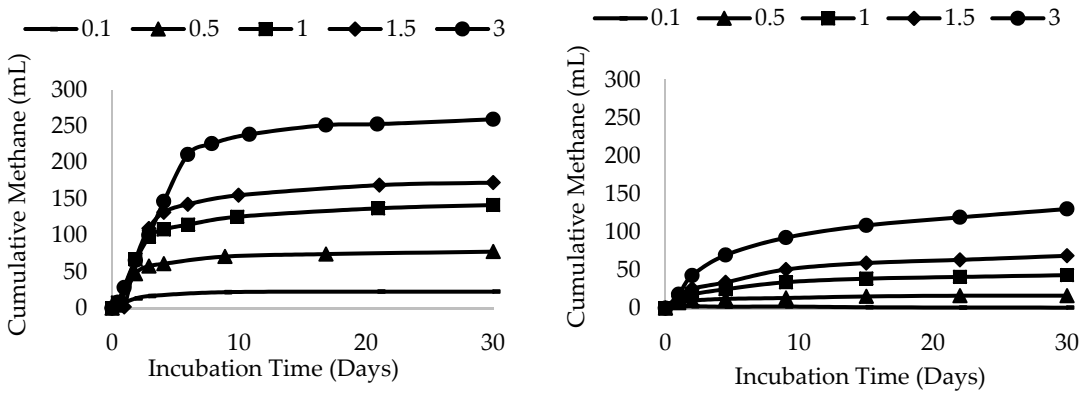

Figure 4. Left: Primary sludge ratio test: cumulative substrate methane production, **right**: secondary sludge ratio test: cumulative substrate methane production (where 0.1, 0.5, 1, 1.5 and 3 are the g substrate COD/g inoculum VS ratios).

Figure 5 shows the methane yield results for primary and secondary sludge. The methane yield in the primary test was found to be 481 ± 1, 470 ± 1, 495 ± 1, 482 ± 1, and 470 ± 1 NmL CH_4/g VS, and corresponding biodegradability (%) of 60 ± 1, 59 ± 1, 62 ± 1, 60 ± 1, and 59 ± 1. These results were similar to those in literature. As stated by Parkin and Owen [7], primary sludge from the primary clarifier is comprised of natural fibers, fats and other solids and has a high biodegradability (69%), reporting typical values in literature of 40–60% reduction in COD and 40–70% reduction in VS [7]. The methane yield of the secondary sludge was 45 ± 1, 166 ± 1, 218 ± 2, 230 ± 2, and 218 ± 1 NmL CH_4/g VS, and corresponding biodegradability (%) of 8 ± 0, 29 ± 1, 39 ± 2, 41 ± 2, and 39 ± 2, for SIR 0.1, 0.5, 1, 1.5, and 3. In literature secondary sludge or waste activated sludge (WAS) is reported to be half as digestible as primary sludge with biodegradability ranging from 30–50% due to the microbial cells

that are often hardly biodegradable causing the degradation kinetics to act slowly [7]. It is important to note the reduction in accuracy as the SIR decreased below 1.0, which is underestimation due to a combination of factors. One would be due to the small volume of secondary sludge added into each serum bottle. As the volume of the substrate was lowered, the secondary sludge had very little to offer the micro-organism, and from having a high headspace volume in relation to the liquid volume lower gas flows and more influence of the initial head space gas [10,42]. As stated by Elbeshbishy et al. [40], having too low SIR may prevent induction of the enzyme necessary for biodegradation. In addition, there is the measurement inaccuracy due to little amount of biogas produced, which would affect the conversion and calculation of the methane yield resulting in significant underestimation. This was observed when the total methane produced by the test bottles for 0.1 and 0.5 generated 2 and 16mL of CH_4 after the blank was subtracted. In comparison, test bottles for primary sludge at 0.1 and 0.5 with the blank subtracted produced 21.1 ± 0.6 and 71 ± 2 mL CH_4.

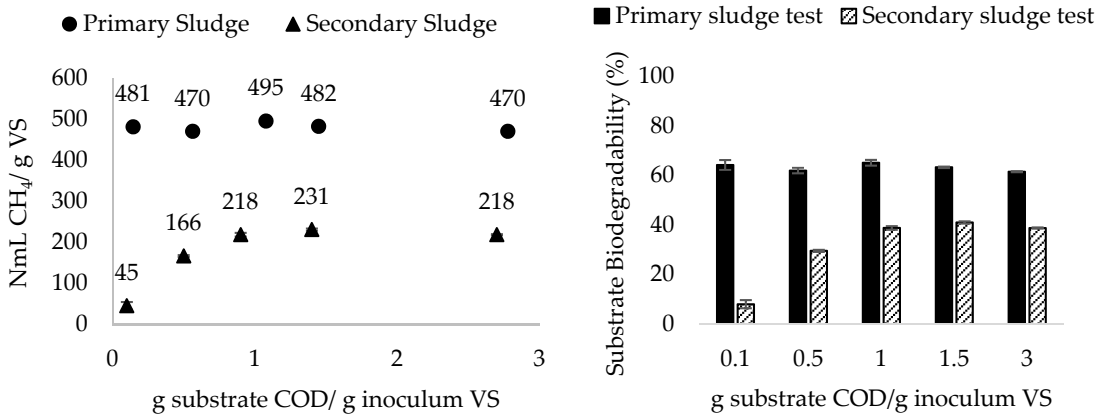

Figure 5. Left: Primary and secondary sludge ratio test methane yield results. **Right**: primary and secondary sludge biodegradability results for different SIR.

Although the most common trend reported was an overestimation of BMP values as the SIR decreased, the substrates used in the experiments appeared to be high in organic content. In the comparison, an underestimation of BMP values could be the case for substrates with very low COD and solid content, therefore requiring higher SIR ratios to be used than wastewater with high organic contents. It is recommended that for substrates with low organic content, with a history of being difficult to digest, SIR should be designed at higher ranges compared to substrates with high organic content and readily biodegradable. In this study, SIR above 1g COD/g VS should be used to determine the BMP values for secondary sludge, while SIR for primary sludge can be lower than 1:1, but it is not recommended. A minimum of three different substrate to inoculum ratios be tested for every new substrate. Additional tests are required to observe the accuracy of BMP values at higher range of F//M values (>3) to observe overloading effects.

To observe the possible impacts the SIR can have on measured kinetic constants, the methane yield curves for primary and secondary were analyzed. The methane production rate constant for a BMP serum bottle experiment was calculated using the following equation, where k is the first order kinetic constant (per day), t is the digestion time (days), and BMP (∞) is the ultimate methane production at the end of the test [40].

$$\mathrm{BMP(t)} = \mathrm{BMP}(\infty) \times (1 - \exp(\mathrm{k} \times \mathrm{t})) \quad (11)$$

MATLAB was used to find the value of k by minimizing the sum of squared differences between the experimental and calculated values. Figure 6 shows the kinetic modelling of the primary and secondary sludge ratio tests. Figure 7 shows there were significant variations between the kinetic constant values between primary and secondary tests. Both experiments k values decreased as the loading rates increased. Primary sludge kinetic values ranged from 0.21 to 0.51, while secondary sludge ranged from 15.2 to 0.151. The kinetic constants for the secondary sludge tests below 1:1, had

greater variation because the substrates were added in small volumes to the inoculum, and were quickly converted to methane. As a result, the accuracy of modelling 0.1 and 0.5 SIR methane yield curves decreased, with R^2 values of 0.49 and 0.96. Kinetic values found in BMP tests should be used with caution, in predicting the kinetic behaviour of continuous digesters. There is the possibility that basic kinetic models over-simplify the dynamics of rate-limiting step, not considering the various conditions in a continues digester operation such as wastewater characteristics, hydraulic loading [61].

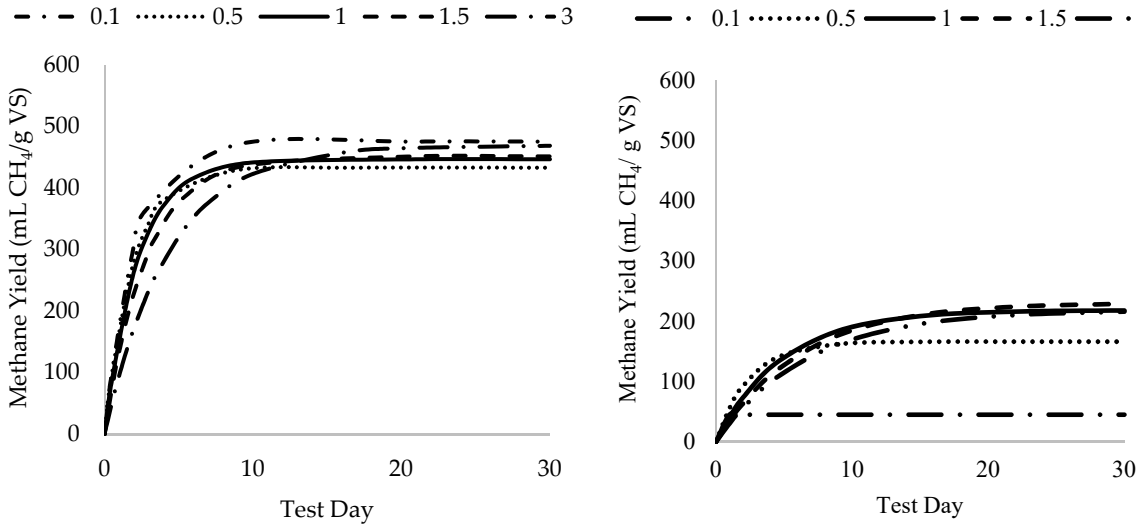

Figure 6. Left: Primary sludge ratio test kinetic modelling of methane yield, **right**: secondary sludge ratio tests kinetic modelling.

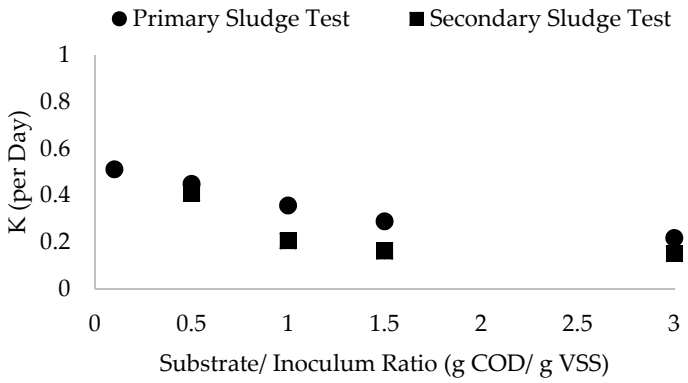

Figure 7. Relationship between methane production rate constant and substrate/inoculum ratio.

There are two general rules for narrowing down the SIR selections. One is the recommendation that for easily biodegradable substrates where rapid accumulation of fermentation intermediates such as VFA could inhibit anaerobic digestion, the inoculum volume should be greater than the substrate or a SIR less than or equal to 0.5 should be applied to minimize the possibility of acidification or inhibition problems (for instance SIR of 0.5 or 0.25) [71]. The second rule is that for substrates that have a high content of non-readily biodegradable organics, a SIR higher than 0.5 should be applied. But, regardless of these rules of thumb, a series of SIR for a new substrate should be tested in order to obtain a reliable BMP values [41,68–70].

3.3.2. Managing Potential Biogas Production

During the period between two subsequent re-equilibrations (gas measurements and gas wasting), the serum bottles are pressurised from gas production. As shown in Figure 8, depending on the organic content of the substrate, a test with highly biodegradable substrate may require more frequent

re-equilibrium/gas releases than a non-biodegradable substrate. For operation purposes it is helpful to predict the estimated volumes of generate biogas for scheduling inspects. The COD to methane conversion ratio, allows for the prediction of the volume of generated biogas. Using 1 g COD = 0.395 L CH_4 for conditions at 35 °C, it is important to balance the liquid volumes of each solution added to avoid the total biogas per day exceeding the headspace volume—leading to over pressurization and requiring increased gas releases. It is recommended that at least 100–200 mL CH_4 or 250–400 mL biogas (assuming 60% CH_4) be produced, allowing for a volume of 10 to 60 mL of biogas to be collected per extraction time. This is important for accurate manual syringe readings and acquiring enough biogas to be processed by the GC.

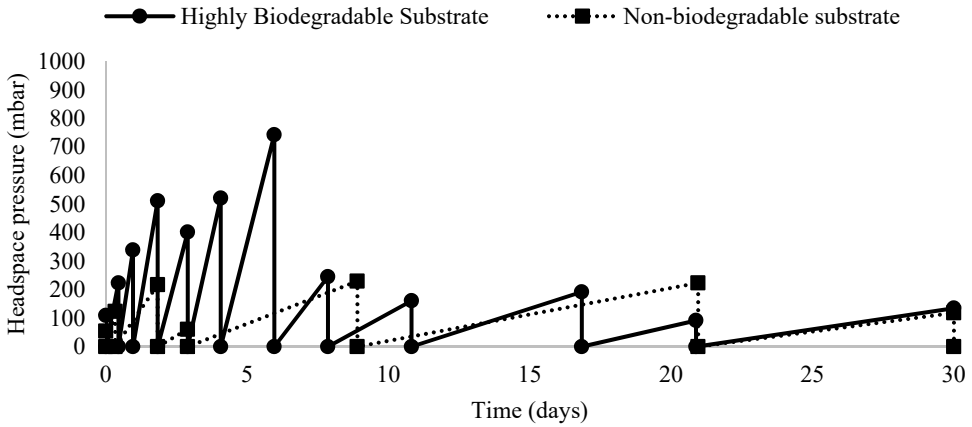

Figure 8. Example of headspace pressure releases for a highly biodegradable and non-biodegradable substrate.

3.3.3. Headspace to Total Solution Volume

The headspace is defined as the non-liquid volume in the serum bottle after filling with testing materials and inoculum. Ratios of the headspace to total bottle volume (160 mL) range from 30 to 70% in reported BMP tests. Normally, the headspace should be larger than the expected maximum produced biogas volume in the first day as it is important to avoid the bottle becoming over pressurized due to the production of biogas and increased temperatures.

3.3.4. Reactor VFA/Alkalinity Ratio

The VFA/alkalinity, as stated by Feng et al. [72] has three critical levels to assess the stability of anaerobic digestion, where (1) <0.4 stable; (2) 0.4–0.8, some instability will occur, (3) >0.8 significant instability [72]. Therefore, during planning stage, it is recommended that the operator adjust the inoculum and substrate volumes for the final solution to be below the first critical level.

3.3.5. Guideline Recommendations

As discussed in the above sections, accurate BMP tests need proper design of the testing parameters to achieve balanced acidification and methanogenesis reactions so that the BMP results can reflect the ultimate methane yield and biodegradability of the substrates. Table 5 shows an example of the design of key BMP parameters used for testing the primary and secondary sludge sampled from the Guelph WWTP. Since the primary and secondary sludges had different properties, the liquid volume of the seed and substrate were different for both tests in order to meet the desired SIR. In order to determine a proper SIR for given sludge properties, as discussed in Section 3.3.1, a series of BMP tests needs to be conducted to assess the effect of SIRs on the methane production. The determination of the total and substrate volumes should consider the total biogas production (Section 3.3.2), headspace to total solution volume (Section 3.3.3), and GC measurement requirement (Section 2.4.1). The total solution alkalinity of the mixed solution or VFA/alkalinity ratio is important to maintain a stable pH condition.

For the anaerobic digestion of wastewater sludge, alkalinity is produced by breakdown of proteins to NH_3 which reacts with CO_2 to form NH_4^+ and HCO_3^-. The accumulation of VFA in the BMP bottles, which will consume alkalinity and cause pH to drop, could inhibit methanogenesis reactions. As stated in Section 3.3.4, it is recommended that the mixed solution have an alkalinity equal to or higher than 3 g $CaCO_3$/L or the VFA/ALK be less than 0.4. It should be kept in mind that the values shown in Table 5 are only examples we determined for the primary and secondary sludge from the Guelph WWTP. These values may not be suitable for other substrates. The optimal SIR, substrate/inoculum volumes, and predicted biogas production will depend on the characteristics of the organic content of substrate.

Table 5. BMP test design for primary and secondary sludge from the GWWTP.

Section	Parameter	Units	Recommendations	Primary Sludge Test		Secondary Sludge Test	
				Blank	Substrate	Blank	Substrate
Volume	Sludge	mL	—	43	43	30	30
	Water	mL	—	10	0	30	0
	Substrate	mL	—	0	10	0	30
	Total Volume	mL	50–120	53	53	60	60
	Headspace	%	30–70	67	67	63	63
SIR	Sub COD g/AD VS g	—	~1	—	1.00	—	0.98
	g VS/ g VS	—	~0.5	—	0.50	—	0.54
Re-equilibrium Period (Assuming 100% Biodegradable)	Predicted Methane Gen.	mL	—	—	165	—	112
	Biogas Gen.	mL	300–500	—	274	—	187
	Max Biogas Production Per 5 Days	mL	—	—	55	—	37
Digester Health	Total Alkalinity	g $CaCO_3$/L	>3	3.87	4.31	2.39	2.43
	VFA/ALK	—	<0.4	0.000	0.062	0.000	0.028

3.4. Execution of BMP Syringe Test

The experimental procedure is split into three sections: (1) start-up, (2) biogas monitoring, and (3) final testing. The start-up stage starts by heating up the seed and substrate to the working temperature. The solution volumes determined from the test design are then added into triplicate groups of serum bottles. The headspace of each bottle is then flushed with nitrogen, and immediately capped with rubber stoppers and sealed with aluminum crimps to make sure the stoppers do not fall out. The bottle are placed in the incubator and after 1 to 2 h, the gas composition of each bottle are measured to ensure the absence of oxygen. Biogas monitoring stage consists of repeated gas sampling, gas composition measurements and gas wasting periodically until the methane production had leveled off. This stage usually lasts longer than 30 days. Final testing stage is the opening of the bottles and measuring the contents for insights into the solution health, solids concentration reduction and mass balance. Table 6 outlines the key steps and the times estimates for each stage.

Table 6. Execution stages and steps for manual operation of BMP serum bottle test.

Stage	Step	Time	Process	Remarks
Start-up	1	1–2 h	Warm liquids to 35 °C	Active inoculum, test material, control solution.
	2	0.1 h	Pour contents into vials	
	3	0.1 h	N_2 Flushing	Every bottle's headspace was immediately flushed with 100% nitrogen gas to remove any oxygen.
	4	0.1 h	Seal bottles and Store at 35 °C	Cap bottles with rubber stoppers and sealed with an aluminum crimp. Place bottles in the incubator at 35 °C.
	5	0.1 h	Re-equilibrate	After 1 to 2 h and measure the gas composition.
Biogas Monitoring	6	>30 day	Biogas sampling	Shake each serum bottle before the gas is vented. Using a gas tight syringe (equipped with a valve between the needle and opening) collect biogas from at least two of the triplicate bottles. At least 10 mL of biogas are needed for accurate GC measurements. Inject the sample into the GC.
Final Testing	7	0.5–1 day	Open bottles and measure contents	Stop test when the methane production has levelled off and the gas composition is constant.

3.5. Data Analysis

After the methane volumes were recorded and the test had ended, the methane volumes were converted into standard conditions (see Table 7). To standardize the BMP results, the as-measured volumes must be converted to standard conditions (0 °C at 1 atm). This involves compensating for both volume occupied by water vapour (generates over-estimations of 2–8% in the gas volume at ambient temperature range) and thermal expansion effects [10,73]. Normally, volumes are measured at one atmosphere, so no pressure correction is required [73]. The biogas sampling intervals was calculated using the following equation:

$$V_{CH4,n}\ (mL) = ((V_{biogas,\ n} + V_{headspace}) \times \frac{\%_{CH4,\ n}}{100}) - (V_{headspace} \times \frac{\%_{CH4,\ n-1}}{100}) \times (\frac{T_{STP}}{T_{gas}}) \times (1 - \frac{P_{vap}}{P_{gas}}) \quad (12)$$

$$P_{vap} = 10^{8.1962-(\frac{1730.63}{Tgas-39.724})} \quad (13)$$

Where $V_{CH4,n}$ is the methane generation volume (mL) of the mixed liquor; $V_{biogas,n}$ is the biogas generation volume (mL) of mixed liquor; V_h is the headspace volume (mL) of each BMP bottle; $\%_{CH4,n}$ is the current methane percentage of the generated biogas determined by GC; $\%_{CH4,\ n-1}$ is the methane percentage of generated biogas in last sampling time point; T_{STP} is the standard temperature (273.15 K); T_{gas} is the incubation temperature (K) for the BMP test; P_{vap} is the water vapour pressure (kPa); P_{gas} is the pressure of the measured gas (101.325 kPa).

Table 7. Data analysis stages and steps for BMP.

Stage	Step	Process	Remarks
Data Analysis	1	Calculate volume of methane of gas produced	Calculate the volume of methane of gas produced in the interval and then calculate the cumulative net volume of the methane produced over the test period
	2	Calculate the methane yield and biodegradability of test material	Dividing the STP methane volume by the mass of the substrate's solids added into the serum bottle. It was reported as mL CH_4 substrate/g VS initial substrate or as mL CH_4 substrate/g COD initial substrate.
	3	Calculate kinetic rate constant	MATLAB was used to find the value of k by minimizing the sum of squared differences between the experimental and calculated values
Data Rejection	4	Check data for data rejection	If the Relative Standard Deviation (RSD) of the blank or the positive control is >5%, even after applying a statistical test to eliminate a single outlier

The methane yield curves were generated by dividing the normalised methane volumes by the mass of volatile solids from the substrate added into the serum bottles. Next, the data when through a screen process to determine if the test was accuracy. As suggested by Holliger et al. [9], the positive control (cellulose) had a relative standard deviation below 5% and produced a methane yield that was within 85 to 100% of its theoretical value. Now that the test had been checked and passed data rejection stage, the final data was organized for presentation.

3.6. Data Report

Data reporting followed the recommendations of Angelidaki et al. [18]. The authors stated that the goal for data reporting is to present a clear description of the inoculum source, substrate, test conditions, and graphics of the specific methane production for the blank, control and substrate. Table 8 and Figure 9 provides an example used for the characterization of primary and secondary sludge using a substrate to inoculum ratio of 1.0. In addition, the inclusion of the kinetic constant can be included as it may provide insight into the time required to generate the ultimate methane potential [18].

Table 8. Example final data information for primary and secondary WWTP sludge.

Data Set	Parameter	Inoculum (Blank)	Control (Cellulose)	Primary Sludge	Secondary Sludge
Physical and Chemical Characteristics	TCOD (mg/L)	19,000 ± 240	17,400 ± 165	47,055 ± 2991	10,670 ± 254
	SCOD (mg/L)	647 ± 20	—	1945 ± 83	58 ± 6
	TS (g/L)	19.74 ± 0.25	—	36.02 ± 0.22	10.36 ± 0.25
	VS (g/L)	10.92 ± 0.12	14.00 ± 0.01	26.09 ± 0.24	6.63 ± 0.12
	TSS (g/L)	18.40 ± 0.48	—	33.57 ± 0.24	9.32 ± 0.48
	VSS (g/L)	12.17 ± 0.38	—	25.03 ± 0.17	6.55 ± 0.38
	ALK (mg $CaCO_3$/L)	4772 ± 160	—	2333 ± 175	83.6 ± 0
	pH	7.6 ± 0.1	7.4 ± 0.1	6.5 ± 0.1	7.1 ± 0.1
Biochemical Methane Potential Data Temp: 35 °C Mixing: 100 rpm Duration: 30 days SIR: 1.0	Methane Yield (NmL CH_4/g VS)	83	375	495	218
	Biodegradability	10%	85%	65%	38%
	Kinetic First order constant (R^2)	0.0855 (0.994)	0.2296 (0.998)	0.3568 (0.981)	0.1406 (0.98)

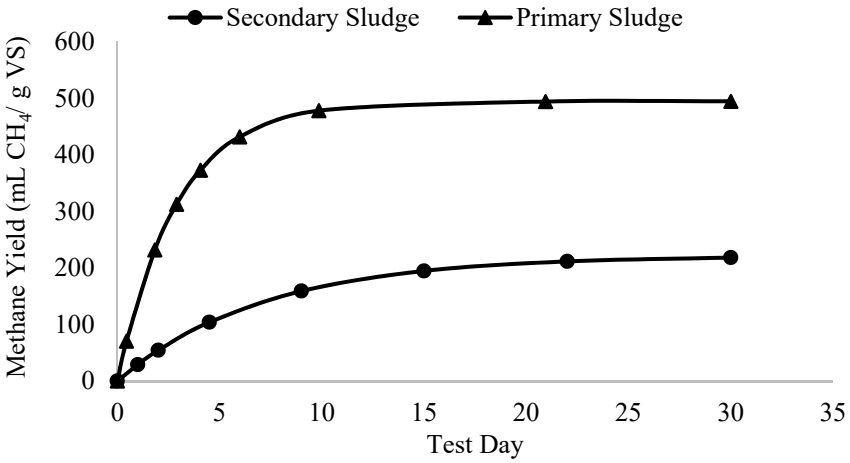

Figure 9. Example final report methane yield graph of primary and secondary sludge from the GWWTP.

4. Future Aspects

To provide perspective, recent research areas being conducted to progress the conventional BMP test were reviewed. So far, these areas include (1) investigations into faster methods to predict the BMP of a substrate, (2) the move towards automated BMP products for standardization, (3) creation of standardized inoculum, and (4) the use of online methane yield data base for data harmonization. The following section will provide a brief description and discussion for each topic.

4.1. Early Prediction of BMP Using Kinetic Modelling

Long test durations are a major drawback of the BMP test. As stated by Da Silva et al. [74] this factor limits the applicability for waste utilities, consulting companies and plant operations how decision-making cannot wait 30 to 100 days. Scientists are researching methods to achieve faster BMP values, such as theoretical determinations and near-infrared spectroscopy. However these methods are limited in providing information about the toxicology and loading rate of the substrate due to the absence of a biological anaerobic degradation process [61]. In addition, these approaches are time independent measurements and offer no information on kinetic degradation. Therefore, early prediction models based on the methane curves. So far Strömberg et al. [61] and Da Silva et al. [74] are the most comprehensive studies to provide laboratory test evidence that shorter test durations could be made using BMP tests.

Strömberg et al. [61], built off the study by Ponsá et al. [75] who showed it was possible to statistically predict the final BMP of a sample with a large enough database. The authors compiled a data base of 138 BMP tests of various substrate types, and 61 different algorithms, to predict the final BMP and required degradation time. The results from the factorial design experiments showed that

the Monod-type, quadratic Monod-type and the first-order model with variable time dependency were able to predict the ultimate methane yield. The statistical prediction estimated the final BMP values and test times by cross-referencing the registered BMP profiles. In a comparison between experimental and predicted results, BMP values after 1, 5, 10, and 15 days made clear that the model predictions improve as the experiment progresses. In addition, by combining the best algorithms, the BMP was predicted with a relative root mean squared error of less than 10% after 6 days of experiment duration. The authors noted that this experiment used only one type of inoculum at mesophilic temperatures, and extensive testing remains at a larger scale (i.e., >138 samples).

Da Silva et al. [74] focused on the first order model to describe the methane yield and kinetic constant rate for easy adaptability. In the study, a threshold time (minimum testing time) was determined using an estimation of the rate constant (using MATLAB 'fitnlm' function). Where early parameter estimation is correlated to the k value, slowly biodegradable substrates ($k < 0.1$ day^{-1}) should have a minimum testing time greater than 15 days, and rapidly biodegradable substrates ($k \geq 0.2$ day^{-1}) have testing times lower than 7 days. In the experiment, three regression results were compared: traditional regression (based on all experimental points from day 0 to day 30 above), threshold regression (all experimental points up to minimum testing time) and balanced threshold regression (using three data points of the initial, threshold and average time between them). The balanced threshold regression improved the quality of parameter estimation, in comparison to threshold regression due to the reduced effect of the initial experimental data. Using the mesophilic BMP test described by Angelidaki et al. [18], common anaerobic digestion substrates such as sewage sludge, primary sludge, pig manure, paunch, blood, and sewage sludge and glycerol mixture, were tested. Although comparing kinetic constant rates to those of other studies is highly variable, the values obtained were within the literature ranges. In comparison to Strömberg et al. [61], Da Silva et al. [74] commented that the minimum testing times for Strömberg et al. [61] were lower. Where Strömberg et al. [61] was able to use a minimum testing time of 4 days for sewage sludge and the authors using 8 and 10 days respectively. The authors stated that threshold time could be reduce if the R^2 criterion was increased from 0.8 to 0.9, however this would increase the occurrence of inaccurate predictions.

Continued research to optimize early prediction methods are recommended. This has significant potential to increase the practicality of BMP tests for waste and water utilities, consulting companies and AD plant operators. In addition, the development may become accurate, with the increased use of automatic methane potential tests as they standardized results.

4.2. Automated BMP

The Automated Methane Potential Test System (AMPTS) II, developed by Bioprocess Control, that has been gaining popularity in anaerobic biodegradability studies for its ability to provide automatic and real-time measurements, recording and reporting of biogas production [10,13,14,22,24,30,31,42,61,76–79]. This tool allows up to 15 test vials (500 mL) in a 35 °C for kinetic information, specific methogenic activity assays and residual gas potential analyses. Using volumetric gas measurement technique, produced biogas is led through flexible tubing to a scrubbing reactor [24]. The acid gases are trapped by an alkali solution and the remaining biogas is passed into the measuring cell containing a gas counter based on the liquid displacement. As the gas bubbles generate impulsions, the volume is recorded and translated into NmL CH_4/g VS by AMPTS v5 software [77].

In comparison to conventional gas measuring systems such as manometer, water column or gas bag, the automatic system is time and labour saving. Wang et al. [23] compared the workload of each BMP test by different experiment setup. By separating a BMP test into three work stages (1) time for inoculum and substrate addition, (2) time for experimental follow up gas volume and composition analysis during incubation, (3) data management and interpretation. The total workload (min/sample) was 540, 220, 220, and 40 for the manometer, water column, gas bag and AMPTS II. In comparison to manual manometric method is simpler to operate. It is predicted that in the future standardization

of the experimental setup, it is inevitable that the BMP method will be automated system due to minimized human error and workload demand, analytical precision, and a standardization of data interpretation [10,23].

4.3. Standardized Inoculum

The development of a method to produce standardized inoculum for a range of substrate in anaerobic digestion batch tests are estimated to increase reproductivity [80]. As of 2017, the Hamburg University of Technology (TUHH) are researching long term preservation methods for anaerobic inocula. Testing results showed that inocula resuspended after preservation high recovery of expected methane production. But, the authors reported a 7–10-day lag phase of methane production possibly due to the damage of microorganism by the preservation method. Further investigations on the optimize preservation process are required [80].

4.4. Online Methane Yield Data Base and Meta-Analysis

Online infrastructure is the next step for published BMP results data harmonization, integration and analysis of overlapping data. In 2015, Murovec et al. [81] developed an online community supported methane yield database. This hierarchically organized, curated and community supported collection of reported methane yields represented the largest publicly available collection with 1164 methane yield entries (15,749 data points) by 71 parameters and 42 substrate categories. During analysis of submitted methane data, the authors reported significant issues due of no variation of due to the variety of data reporting in published literature, highlighting the importance of standardized methods and reporting for results comparability. Out of all the data information entries, only 5.6% contained all required data and 80% of methane yield entries had uninformative metadata. 17.2% of entries had to be removed because entry data were ambiguous data. In order to improve the methane yield database to be valuable, the authors had three recommendations: (1) there needs to be adjustments made by both scientists and the industrial community in the reporting, (2) the methane yield data sever needs to expand the available data categories and parameters, and (3) there needs to be and advancement of data analysis tools for developing the ability to exchange data in both directions [81].

5. Conclusions

The objective of this paper was to outline an accessible and simplified serum bottle method. Unlike previous methods proposed, this is a very simple laboratory technique, and allows a large number of replicates at different conditions that can be carried out simultaneously to provide comprehensive reproducible results. As of 2018, there continues to be a lack of standardized/universal BMP testing procedure resulting in a lack of comparable BMP values due to the differences in equipment, experimental conditions and procedures. However, the BMP methods continue to evolve and further the elimination of systematic errors. In this study, BMP protocols guidelines and recommendations were reviewed and summarized, which rendered the following conclusions:

1. The substrate to inoculum ratio has been found to be an important design parameter for achieving accurate BMP serum bottle results. In addition, the accuracy of BMP tests could also be significantly affected by selection of blank and control bottles, head spacing flushing, mixing, pH control, and methane production monitoring and correction methods.
2. Kinetic models could be used to predict the methane production based on BMP tests with a reduced testing period but the selection of the model and kinetics could varied widely with different testing conditions and need to be carefully verified.
3. Future aspects of BMP test include further research into time-saving techniques, the use of online database for accessibility, standardized inoculum, and increased use of automated BMP systems for time saving, standardized results and reductions in human error.

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Abbreviations

ALK	Alkalinity
AD	Anaerobic Digestion
BMP	Biochemical Methane Potential
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
CLRS	Continuous Measurements with Liquid Replacement
GC	Gas chromatography
LRS	Liquid Replacement System
CH_4	Methane
SRT	Solid Retention Time
SCOD	Soluble Chemical Oxygen Demand
SIR	Substrate to Inoculum Ratio
TS	Total Solids
TSS	Total Suspended Solids
VFA	Volatile Fatty Acid Concentration
VS	Volatile Solids
VSS	Suspended Volatile Solids
WWTP	Wastewater Treatment Plant

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