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Quantification of leakage in batch biogas assays

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Abstract

Avoiding leaks is essential for accurate measurement of biogas production by batch assays. Here we present a simple method for detecting leaks and correcting results, based on the change in bottle mass during incubation. Three experiments were carried out using pure chemicals, wastewater sludge, and other complex substrates to test and demonstrate the method, and leaks were detected in all three. The frequency and magnitude of leakage was related to headspace pressure and the number of times bottle septa had been punctured. Comparison to an independent estimate of leakage in two experiments showed that the proposed method is accurate. This mass-based approach can generally be used to detect leaks as small as 20% of total biogas or methane production, or lower when biogas production is high relative to the precision of mass measurements. Additional research is needed to improve the sensitivity of the method and to better understand the causes of leakage. Given the potential importance of leaks and the simplicity of leakage measurements, we recommend that this method is always used in batch biogas assays.

Key words: biochemical methane potential, biogas, BMP, laboratory methods

INTRODUCTION

Biogas production is commonly measured in laboratories using batch assays (Angelidaki *et al.* 2009; Raposo *et al.* 2012). These measurements are essential for detecting inhibition and quantifying the methane that could be produced from wastewater sludge and other substrates. Because most methods rely on biogas containment to measure biogas or methane (CH_4) production (Rozzi & Remigi 2004; Angelidaki *et al.* 2009; Raposo *et al.* 2012), avoiding gas leaks is essential for accuracy. Existing protocols recommend both leak checks prior to setting up an experiment and maintaining low headspace pressures, but they do not include any means to check for leaks when measuring biogas production (Owen *et al.* 1979; Angelidaki *et al.* 2009; Holliger *et al.* 2016; VDI 2016). In volumetric and manometric methods, biogas commonly accumulates in bottles that are sealed with septa. Leaks may develop due to repeated puncture of septa by needles. Measurement of CH_4 production from cellulose or other defined substrates could provide an indication of leaks for individual bottles, and possibly an approximate estimate of average leakage, but do not provide direct leak estimates for other bottles. Since leakage is probably sensitive to headspace pressure, leak loss could differ substantially among bottles. Richards *et al.* (1991) described how gravimetric measurements could be used to check for leaks. The method was recently refined (Hafner *et al.* 2015b), but the approach has not been widely applied. In this work we describe a much simpler gravimetric approach to detecting and quantifying leaks, and demonstrate its use. The method can be applied to any biogas assay where biogas accumulates in a bottle over time.

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METHODS

In a typical volumetric or manometric assay, biogas volume or pressure is measured intermittently after several short (ca. 0.5 d to >1 week) incubation periods. The total duration may be 30 d, 60 d, or longer. The only measurements required for leak detection and quantification in the proposed method are the mass of individual bottles before and after each incubation period. A decrease in mass during incubation indicates leakage. The fraction of total biogas lost through leaks can be estimated from the ratio of leak mass loss to total mass loss from the bottle. Three experiments were carried out to test and demonstrate the proposed method. Biogas production was measured gravimetrically in all experiments, and also volumetrically in Experiments 1 and 2. A comparison between gravimetric and volumetric results provided an independent estimate of leakage that was used to evaluate the proposed method.

Experimental

Cellulose, ethanol, and inoculum-only bottles were included in all experiments, in addition to complex substrates. In Experiment 1, at total of five substrates were used (Table S1). Serum bottles had a total volume of 1,100 mL, and received 300 to 500 g of inoculum (total mass) and 1.4 to 3.7 g of substrate volatile solids (VS) (Table S2). Total incubation time was 31 d. Municipal wastewater sludge was the substrate in Experiment 2. Secondary sludge was either raw or previously anaerobically digested, and was treated by thermal hydrolysis in some cases (Haarslev Industries Continuous Hydrolysis System, Denmark) (Table S3). Bottles had a total volume of 320 mL, and received 3.0 to 3.5 g substrate VS (Table S4). Total incubation time was 31 d. In Experiment 3, serum bottles with a total volume of 320 mL contained 150 g of inoculum and 0.7 to 2.8 g of substrate VS (Table S5). Straw, cat food (representing food waste), as well as pure substrates were used (Table S5). Total incubation time was 113 d. All conditions were evaluated in triplicate.

Inoculum was digestate from an anaerobic digester at a full-scale wastewater treatment plant (Ejby Mølle, Odense, DK for Experiment 1 and Næstved Renseanlæg, Næstved, DK for Experiment 2), or digestate from a lab-scale reactor treating secondary sludge (for Experiment 3). When collected from a wastewater treatment plant, inoculum was stored a maximum of five days at 35°C or 37°C with a water trap before use. The VS content ranged from 19.9 g kg^{-1} in Experiment 1 to 32.5 g kg^{-1} in Experiment 3 (Tables S1, S3, and S5). Inoculum pH was 7.26 in Experiment 1, 7.62 in Experiment 2, and was not measured in Experiment 3.

After adding inoculum and substrate (and 1 mL each of a vitamin and a trace element solution following Holliger *et al.* (2016) in Experiment 1), bottles were sealed with new bromobutyl rubber septa (article no. 4408, West Pharmaceuticals, Le Nouvion, France) and flushed with N_2 for at least one headspace exchange, weighed twice (to avoid data recording errors (Hafner *et al.* 2015b)), and placed in an incubator at (35°C for Experiment 1, and 37°C for Experiments 2 and 3, $\pm$1°C). Because of a possible inventory mistake (mixing used and new septa), it is possible that some of the septa had been previously used in Experiment 2. Weighing was done with a Mettler PJ3600 electronic balance (Zurich, CH). This is an older unit (last produced in 1992) with a readability of 0.01 g for masses under 600 g, and 0.1 g otherwise. Incubation intervals were one d initially, and longer as biogas production slowed. Maximum headspace pressure depended on rates of biogas production, headspace volume, and sampling frequency, and varied among the experiments. It was lowest in Experiment 1 (measured maximum of ca. 120 kPa, gauge), and highest in Experiment 3 (up to nearly 500 kPa, gauge). At the end of each incubation interval, bottles were removed from the incubator and placed on a lab table at room temperature, three at a time. The same sequence of steps was always used to measure biogas production at the end of each incubation interval:

1. The bottle was mixed by swirling, avoiding contamination of the septum with reacting material
2. The bottle was weighed

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3. Headspace pressure was measured using an electronic manometer
4. Biogas was removed using one or more syringes (simultaneously) until pressure was 0 ± 1 kPa
5. A ca. 10 mL biogas sample was collected from the removed gas and injected into an evacuated glass vial
6. The bottle was again weighed

The entire process took 1 to 3 minutes per bottle. Room temperature was recorded during each measurement event. The temperature of the wall of the bottle in contact with the gas in the headspace was occasionally measured using an infrared thermometer (no. 620-2203, VWR, Radnor, PA, USA). For volume measurement, the temperature of a 1 L syringe was occasionally measured using the same thermometer, and was found to be within 1°C of room temperature. The manometer was a Dwyer 475-7-FM (Michigan City, IN, USA) with a range of 0 to 700 kPa (gauge) or a Sper Scientific 840082 (Scotsdale, AZ, USA) with a range of 0 to 200 kPa (gauge). In Experiment 3, volume and pressure measurements were not made for every bottle after each incubation interval, and so only gravimetric measurements were used.

Needles were 21 gauge (0.8 mm) stainless steel with a beveled tip (BD Medical, Franklin Lakes, NJ, USA). Septa were punctured twice during flushing and two to four times during biogas measurement at the end of each incubation interval. Biogas composition was determined by gas chromatography using a thermal conductivity detector (Agilent 7890A, J and W 113-4332GS column, oven temperature 250°C). Gas standards (70% CH_4, 30% CO_2) were included in every run. Two or three bottles with only tap water were included in each experiment. Water-only bottles had a similar mass as sample bottles and were weighed once after each incubation event to check scale stability and quantify the precision of mass loss measurements. The septa sealing these bottles were never punctured, and mass loss was expected to be null.

Data processing

Biogas production was calculated volumetrically or gravimetrically using the cumBg() function in the biogas package Hafner *et al.* (2015a) in R (R Core Team 2017). Biogas temperature for volumetric calculations was assumed to be the mean room temperature. Methane concentration was normalized so the sum of CH_4 and CO_2 concentration was 1.0 (Richards *et al.* 1991; Hafner *et al.* 2015a, 2015b). For the gravimetric method, which is not very sensitive to biogas temperature or pressure (Hafner *et al.* 2015a, 2015b), temperature was taken as 33°C, which was between typical bottle wall measurements and incubator temperature, and headspace pressure was set to a fixed value of 150 kPa (absolute). The difference between gravimetric and volumetric measurements of biogas and CH_4 production was used to independently estimate leakage to evaluate the proposed method. Gravimetric results were taken as the true values. To assess the relationship between headspace pressure and leakage, maximum possible headspace pressure during an interval was calculated based on gravimetric estimates of biogas volume and headspace volume, correcting for temperature and water vapor (Hafner *et al.* 2015a, 2015b).

Leak detection and quantification

In all experiments, the total mass of each bottle was measured at the start after bottles were sealed and flushed (m_a), after each incubation interval but before biogas removal (m_b), and after removing biogas (m_c). With these measurements significant leaks cause mass loss from m_c at time $t-1$ to mass m_b at time t as in Equation (1), or, for the first incubation interval, from m_a to m_b at time 1, as in Equation (2).

$$l_t = m_{c,t-1} - m_{b,t} \tag{1}$$

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$$l_1 = m_a - m_{b,1} \tag{2}$$

In Equations (1) and (2), l is the apparent leak mass and m is total bottle mass. Depending on scale precision and readability, l will contain significant random or even systematic error. But where mass drops by a quantity significantly higher than the variability of the water-only bottles, this is strong evidence of a leak. Leak mass detection limit can be estimated for each experiment as 3× the standard deviation of mass measurements on one or more water controls. This estimate should be compared to the largest absolute value of the mass change in a water-only bottle and the largest apparent increase in mass of any bottle (apparent mass increase is due entirely to measurement error), and adjusted upward if necessary. Leakage during a complete incubation (all intervals) is calculated as the sum of interval values (Equation (3)).

$$l_{total} = l_1 + \sum_{i=2}^{k} l_i \tag{3}$$

To determine the detection limits for the complete incubations, the interval limit may be multiplied by $\sqrt{k}$, where k = the number of incubation intervals, to reflect the higher error for the sum of interval values (Brown & Berthouex 2002). This approach was used here. Alternatively, if water-only bottles are weighed twice after each incubation interval (as with sample bottles), apparent total leak loss can be calculated as with samples, and the standard deviation of these observations can be used to directly determine a detection limit.

To quantify and correct for leaks, both the total leak mass and the total mass loss during the complete incubation (Δm) are needed.

$$\Delta m_{total} = m_a - m_{b,k} \tag{4}$$

In Equation (4), k indicates the final measurement interval. Relative error (fraction of total biogas produced lost through leakage) is then given by Equation (5).

$$e_{rel} = \frac{l_{total}}{\Delta m_{total}} \tag{5}$$

This approach assumes that there is no correlation between biogas composition and the timing of leaks. Depending on the size of the calculated error and precision of the mass measurements, users can choose to correct measured biogas production or discard data. Correction of volumetric or manometric estimates of biogas or CH_4 volume can be made with Equation (6).

$$v_{leak} = \frac{e_{rel} v_{meas}}{1 - e_{rel}} \tag{6}$$

In Equation (6), v_{leak} is the volume of biogas or CH_4 lost through leaks, and v_{meas} is the measured biogas or CH_4 volume.

RESULTS AND DISCUSSION

Experimental results

Leaks were observed in all experiments, and their frequency and magnitude varied within and among experiments (Table 1). Leak estimates made with the proposed method were close to those calculated by comparing volumetric and gravimetric results, confirming the accuracy of the proposed approach.

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Table 1 | Total incubation leakage estimates, methane production measurements, and other details for a subset of bottles from Experiments 1 and 2

			Methane[b] (mL)					Leakage[d]		
Experiment	Bottle key	Substrate VS[a]	Grav.	Vol.	Diff.	Max. pres. (kPa)	Mass loss[c]	Mass (g)	CH_4 (mL)	CH_4 (%)
1	A2	3.14	1,477	1,476	1	103	3.6	0.0	0.0	0.0
1	B2	3.01	1,439	1,414	25	114	3.7	−0.1	−37	−2.7
1	C1	2.73	1,900	1,808	91	110	3.7	0.2	103	5.4
1	L2	1.35	814	547	266	53	2.1	*0.8	337	38.1
1	C5	3.23	1,981	1,083	898	91	4	*1.9	980	47.5
2	41	3.36	1,055	1,065	−11	142	1.95	0.01	5.0	0.5
2	11	3.26	1,316	1,220	97	139	2.23	0.12	69	5.4
2	B3	3.16	988	914	74	123	1.86	0.16	86	8.6
2	B1	3.21	999	916	83	126	1.88	*0.19	103	10.1
2	61	3.27	1,025	732	293	93	1.94	*0.51	261	26.3

Selected bottles include examples of no or very low leakage, non-detectable leakage, and high leakage.
Notes:
[a]Substrate volatile solids.
[b]Total methane production determined using the gravimetric or volumetric method.
[c]Total mass loss over all incubation intervals.
[d]Total leakage estimates based on mass change during incubation intervals, calculated using Equations (3), (5) and (6). Mass was calculated from Equation (3), fraction of total CH4 by Equation (5), and CH4 volume by Equation (6). Mass values with an * were above the experiment detection limit.

In Experiment 1, the apparent mass change in water bottles over incubation intervals ranged from −0.1 to 0.1 g. The standard deviation of these apparent changes (s) was 0.041 g, resulting in a leak detection limit of 0.12 g ($3s$). Most observations of sample bottles showed no measurable mass change (142 observations, or 75%). But for two of 21 bottles, mass loss over one or more individual incubation intervals exceeded the leak detection limit (Figure 1). Over the entire 31 d incubation, the detection limit was 0.37 g (based on 9 incubation intervals), and a total of four bottles had detectable leaks over the entire incubation (Figure 2, Table S2). The four bottles that leaked did so in three different ways (Table 1 and Table S2). One (C5) leaked large volumes during all incubation intervals except the first, regardless of headspace pressure. A second (C4) had one large leak during the first incubation interval, when calculated maximum pressure reached the maximum observed of 158 kPa (gauge). The remaining two leaking bottles leaked at low rates during 4 (I3) or 8 (L2) of 9 intervals, with leak mass always below the interval detection limit. For both incubation intervals (Figure 1) and the total incubation (Figure 2),

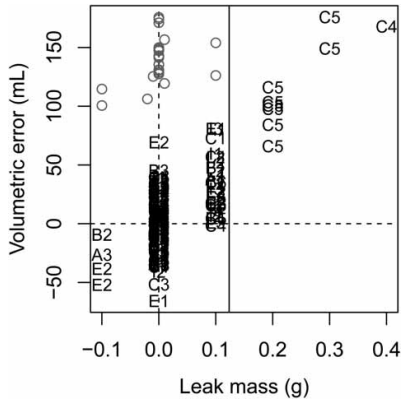

Figure 1 | Apparent mass loss during individual incubation intervals and error in volumetric measurement of methane for Experiment 1 (based on a comparison to gravimetric results, positive for underestimation by volumetric). The solid vertical lines show detection limit (0.12 g). Plotting symbols give the substrate (letter) and replicate (number) (see Tables S1 and S2 for more details). Gray circles show variation in apparent mass change for water controls (y axis is not applicable).

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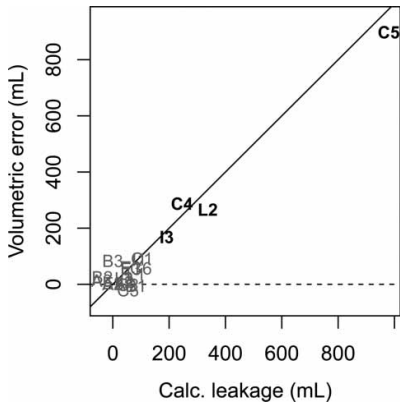

Figure 2 | Comparison of methane leakage (calculated from Equation (6)) and error in volumetric measurements of methane production determined by comparison with gravimetric measurements for Experiment 1. Plotting symbols are described in caption to Figure 1. Symbols in gray had leak mass loss below the detection limit.

leak mass was correlated with error in volumetric measurements. For bottles with detected leaks, calculated volume of methane lost by leakage based on Equation (6) and volumetric measurements ranged from 191 mL to 980 mL (10% to 48% of total CH_4 volume) (Figure 2). Calculated leak volume was similar to volumetric error (Figure 2).

In Experiment 2 apparent mass change of water controls ranged from −0.02 to 0.03 g (Figure 3), and s was 0.013 g, for an interval detection limit of 0.048 g (3 s). A total of 41 observations from 24 bottles showed leak mass loss above the detection limit, with a maximum value of 0.22 g (Figure 3). For the entire incubation, the leak detection limit was 0.17 g (based on 12 incubation intervals, which ranged in number from 7 to 12). Of the 33 bottles, 14 (42%) showed detectable leakage, and 11 of these lost >10% of the biogas produced (Table S4). Detected leakage ranged from 73 to 265 mL, or 10% to 26% of biogas produced over the total incubation (Table S4). As in Experiment 1, leak loss was correlated with error in volumetric measurements for both incubation intervals (Figure 3) and for the total incubation (Figure 4). Also as in Experiment 1, total leak loss (calculated using Equation (6)) and error in volumetric measurements were very similar (Figure 4). But leakage followed a different pattern than in Experiment 1. Leaks were much more frequent, although none were apparent until nearly 5 days into the incubation, after four incubation intervals. Afterwards, leakage seemed to become more frequent, perhaps due to accumulating damage to septa from needles

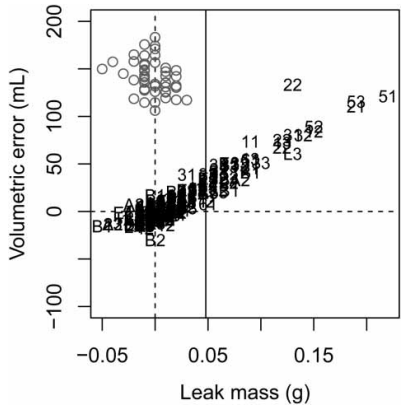

Figure 3 | Apparent mass loss during individual incubation intervals and error in volumetric measurement of methane for Experiment 2 (based on a comparison to gravimetric results, positive for underestimation by volumetric). The solid vertical lines show detection limit (0.048 g). Plotting symbols are the bottle keys (see Tables S3 and S4 for more details). Gray circles show variation in apparent mass change for water controls (y axis is not applicable).

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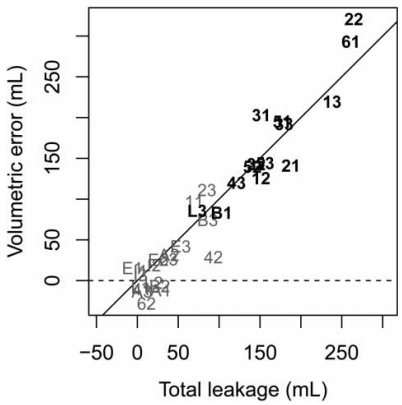

Figure 4 | Comparison of methane leakage (calculated from Equation (6)) and error in volumetric measurements of methane production determined by comparison with gravimetric measurements for Experiment 2. Plotting symbols are bottle keys (see Tables S3 and S4). Symbols in gray had leak mass loss below the detection limit (0.17 g).

(Figure 5). Headspace pressure was not high; the maximum measured value was 150 kPa, and the highest calculated maximum was 170 kPa (both gauge). But leak occurrence and magnitude also appeared to be related to pressure (Figure 5).

In Experiment 3, $s = 0.021$ g for water controls and the interval detection limit was 0.064 g (3 s). A single apparent mass gain of 0.09 g was omitted from these calculations, since it was much larger than any other observation, and thought to be due to a data recording error. For the total incubation, the detection limit was 0.18 g (based on 7 incubation intervals). Headspace pressures reached much higher values than in Experiments 1 and 2 (up to 498 kPa, gauge), but only four of 21 bottles showed leakage (a single leak each) over the first 23 d. However, over the next, extended, incubation interval (90 d), 17 of 21 bottles leaked. Detected leaks ranged from 109 to 370 mL of CH_4, or 9.4% to 30% of biogas production over the complete incubation (Table S6). (Absolute leak volumes were calculated from gravimetric results, since volumetric measurements were not made.) The magnitude of leakage appeared to be related to headspace pressure, with higher pressure associated with larger leaks, when leakage did occur.

Precision of leak measurements

Although gravimetric measurements of biogas production may be more accurate than volumetric or manometric measurements since leakage does not affect results, sensitivity is generally poorer. The

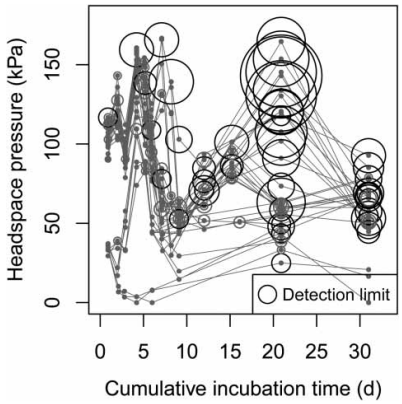

Figure 5 | Leak occurrence and magnitude versus time and headspace pressure for Experiment 2. Lines connect points from individual bottles, and circle size is proportional to leak mass. Gray circles show apparent mass change below the detection limit (0.048 g).

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leak results presented here are based on measurements that were close to method detection limits (Table 1). Low sensitivity is, in general, a limitation of the proposed method. Considering the calculated detection limits and the readability of the scale that was used, the minimum detectable mass loss was 0.4, 0.14, and 0.19 g for experiments 1, 2 and 3, respectively. Expressed as a volume of CH_4 (assuming a CH_4 concentration of 70%, using the mass2vol() function (Hafner *et al.* 2015a)), these are 246, 86, and 117 mL. In Experiment 1, total CH_4 production ranged from 400 to 2,500 mL (Table S2), and only for the single bottle with the highest production would detection of a 10% leak be possible. However, loss of 20% of total biogas production could be detected for most bottles (15 of 21). The smaller bottles (and less substrate) used in Experiments 2 and 3 produced less biogas but the complete incubation detection limits were one-half to one-third of the limit in Experiment 1. In Experiment 2, a 10% leakage loss would be detectable for most bottles (24 of 33), and a 20% loss would be detected for nearly all (29 of 33). In Experiment 3, a 10% leakage loss would be detected for only 6 bottles, but a 20% loss would be detected for most (17 of 21). Sensitivity of the method could almost certainly be improved with better equipment. More precise scales exist (e.g., Ohaus Explorer, 1.1 kg capacity, 1 mg readability, which is 100-fold better than the measurements from Experiment 1), and their use could significantly improve the sensitivity of the method. A draft shield may further reduce variability in measurements (all weighing in these experiments was done using an open balance with no draft shield).

The difference between bottle and room temperature during weighing could contribute to error in mass determination by creation of air currents. For best results, the two temperatures should identical (Ewing 1997). This effect could affect both precision and accuracy, since mass c described in Section 2.3 may be determined a few minutes after mass b, and therefore a systematic bias may be present. We checked for an effect with a subset of 6 bottles from Experiment 1 by weighing directly after removal from the incubator (temperature of 35°C) and then after 10 min at room temperature (20°C). No variation was seen, although all but one bottle weighed more than 600 g, so readability of the scale was 0.1 g and small effects would not have been detected.

Assuming that total relative leakage of biogas and CH_4 are equivalent (e.g., as is done in Equation (6)) implies that leak occurrence and magnitude are not correlated with biogas composition. This assumption may not be accurate. For example, if leakage occurred only during the latest incubation intervals (as for some of the bottles in Experiment 3) when CH_4 concentration is typically higher than average, Equation (6) would underestimate CH_4 loss and overestimate biogas loss. However, this error will probably be small compared to random error due to limited precision of mass loss measurements.

Addressing the problem of biogas leaks

Since methods vary among laboratories, we cannot be sure that the leakage frequencies found in Experiments 1, 2, or 3 are typical. Regardless, it is clear that leaks are at least possible. How can leakage be prevented? One option is to simply use a gravimetric approach for measuring biogas production (Hafner *et al.* 2015b). Unlike all alternatives, leaks do not affect results. The mass measurements described in Section 2.3 are the only ones required (and mass b is optional), although calculations are more complicated than those described here. For all other methods (volumetric, manometric, and the method of Hansen *et al.* (2004)) we recommend that leaks are measured using the method described above, and data from individual bottles either corrected or discarded if leaks are detected. As shown in the three experiments presented here, it is not reasonable to assume that leakage is negligible. Unfortunately, the method described here is only applicable to approaches where biogas accumulates. It cannot be used when biogas volume is measured continuously as it is produced (e.g., using the AMPTSII system (Bioprocess Control, Lund, Sweden) or the Ritter Biogas Batch Fermentation System (Dr.-Ing. RITTER Apparatebau GmbH & Co. KG,

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Bochum, Germany)). Even where leakage can be measured, precautions should be taken to avoid leaks. But exactly what these precautions are is not clear at this time. Minimizing headspace pressure can reduce the risk of leaks, but not if it is done by increasing sampling frequency, and thus the damage to septa. What pressure should be taken as a safe maximum is not clear. Holliger *et al.* (2016) recommended pressures below 300 kPa (absolute) to avoid encouraging CO_2 dissolution and avoid explosions. Owen *et al.* (1979) recommended a maximum gauge pressure of 0.5 atm (ca. 150 kPa absolute) to avoid leakage. A limit of 150 kPa in Experiments 1 through 3 would have eliminated many but not all of the observed leaks, assuming they were indeed sensitive to pressure as the results suggest. Direct measurement of headspace pressure may not be the best approach for determining if the selected maximum has been exceeded, since this value may be significantly lower than the actual maximum during an incubation interval when leakage does occur. Instead, gravimetric measurements of biogas volume can be used to estimate maximum pressure (see Section 2.2). Given a simple, accurate method for detecting leaks, additional work is now needed for developing protocols that minimize leakage.

Conclusions

Leaks can be detected and accurately quantified in batch biogas assays by simply weighing bottles before and after each incubation period. Given the simplicity of the method, we recommend that it is included in biogas assays whenever possible. Our results show that both small and large biogas leaks may not be rare. Future work should focus on improving the sensitivity of the method, and identifying causes of leakage and practices that can prevent it.

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