Decomposition and methane production in anaerobic environments: a case study in a methanogenic bioreactor

Emily K. Geoghegan\textsuperscript{1,2}, Zoe Cardon\textsuperscript{1}, Joseph J. Vallino\textsuperscript{1}

\textsuperscript{1}The Ecosystems Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 USA

\textsuperscript{2}Bryn Mawr College, Bryn Mawr, PA 19010 USA
Methane in the atmosphere contributes more strongly to global warming than CO$_2$ per unit, but this potent greenhouse gas is also a promising source of cleaner, renewable energy in place of fossil fuel combustion. Bioreactors house methane-producing microbes that convert feedstocks such as sewage sludge or plant matter into methane biofuel. In this experiment, I tested treatments with the purpose of increasing methane yield in an algae-to-methane bioreactor. In this closed-system bioreactor, algae grown under light in one container feed anaerobic decomposition in a second container called a digester. Nutrients resulting from this anaerobic decomposition in the digester are then recycled back into the algal growth container to fuel growth. I tested whether the addition of 100μM glucose with algae, 100μM acetate with algae, or autoclaved algae to the digester could improve methane yield relative to an unaltered control. I measured carbon dioxide, methane, and dissolved organic carbon at 4 time points over three weeks. Carbon dioxide production was significantly higher in the autoclaved treatment than the other three treatments. Methane production over time in the autoclaved treatment did not vary significantly from the control, but the glucose and acetate treatments produced significantly less methane than the control. While a trend suggests that the autoclave treatment could eventually stimulate a greater methane yield, results are not conclusive due to the lack of replicates at the final measurement point.

Key Words and Phrases
methanogenesis, decomposition, anaerobic digestion, bioreactor, algae, optimization, pretreatment, incubation, glucose, acetate

Introduction
Methane gas contributes more strongly to global warming than CO\(_2\) per unit, but this potent greenhouse gas is also a promising source of cleaner, renewable energy in place of fossil fuel combustion. Methane is 23 times more potent than CO\(_2\) (Renner 2007). Humans release significant amounts of methane gas into the atmosphere through sources such as landfills and livestock production (Kerr 2010). However, methane is also produced naturally in environments such as bogs and swamps where oxygen is scarce and anaerobic decomposition occurs. While potentially harmful as a greenhouse gas, methane production can be beneficial as an alternative energy source when contained in controlled environments (Ajeej et al. 2015).

Methane biofuel production through anaerobic digestion is a promising alternative to fossil fuel combustion due to its small carbon footprint. Methane produced during anaerobic decomposition (anaerobic digestion) can be burned to generate electricity or compressed and used to power motor vehicles (Dioha et al. 2013). Most commercial anaerobic digesters fuel methane production with organic material from plants or wastewater. However, the aim of the coupled Algae-to-Methane (A2M) bioreactor set up by Joe Vallino and Zoe Cardon at the Marine Biological Laboratory in Woods Hole, MA is to create a system that recycles nutrients and produces methane without external inputs.

The A2M bioreactor contains an algal growth chamber and an anaerobic digester. The anaerobic digester in the A2M system ideally remineralizes nutrients for the connected algal growth chamber. Algae grow and reproduce in the algal growth chamber using artificial sunlight. Then, the algae flow into the anaerobic digester where they die from lack of light and oxygen. This dead algal mass provides chemical energy for anaerobic decomposers and fuels anaerobic digestion. Organic material entering the digester ideally undergoes the four steps of anaerobic digestion - hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Veeken et al. 2000). The final step of anaerobic digestion – methanogenesis - ultimately produces methane gas. Remaining dissolved nutrients created during anaerobic digestion flow back into the algal growth chamber to stimulate algal growth and reproduction, and the cycle continues.

However, this process is not currently functioning because methane production is very low. Researchers originally inoculated the algal reactor with a mutant of *Chlamydomonas reinhartii* (CW15) that lacks a cell wall, and therefore should have been more easily digested than algae with tough cell walls. But, microscopic views of the algal substrate indicate that this alga species is no longer present in the algal reactor. It has been replaced by new, unknown
algae with cell walls that likely come from the digester fluid that was inoculated with sewage and anaerobic ground water from Little Pond. This change in algal community may cause a buildup of organic matter in the digester because the current species of algae present cannot be decomposed as easily as the algae that lacked a cell wall. I want to investigate how the A2M bioreactor can achieve optimal methane production despite this shift in algal community.

The decomposition of organic materials is vital for anaerobic digestion to occur (Kangle et al. 2012). Methanogens cannot directly utilize nutrients in complex organic form, so the lack of organic matter decomposition in the digester can limit methanogenesis. I suspect that low decomposition rates may currently limit methanogenesis in the anaerobic digester. By determining how to increase decomposition of organic material in the anaerobic digester, we may be able to increase methane production. I tested three types of treatments previously suggested or shown to stimulate methane production – addition of glucose, addition of acetate, and hydrothermal pretreatment of algal feedstock.

In the past, the addition of glucose and acetate improved methane yield in the anaerobic digester. This past September, 1mM acetate and 1mM glucose were added to the anaerobic digester a few days apart in an attempt to stimulate increased methane production. Methane production gradually increased a few days after the addition of glucose (Figure 1). From these data, I infer that acetate and/or glucose could play a role in increasing methane production in the A2M anaerobic digester once again. Since both acetate and glucose were added around the same time, it is impossible to determine which substance is responsible for the stimulated methane production. I tested the effects of adding these substances separately to determine their individual effects.

Glucose may stimulate methane production because the sugar could act as a “primer” for organic matter decomposition. The ‘priming effect’ postulates that organisms can decompose more complex substances they cannot currently break down if they are given an initial boost of energy in the form of easily metabolized compounds (Jenkinson et al. 1985). Glucose may act as a primer that gives microorganisms in the early stages of anaerobic digestion the energy needed to break down the new algal bodies that possess cell walls.

Acetate can directly affect methanogenesis, particularly if acetoclastic methanogenesis is a dominant pathway. I suspect that the addition of acetate might increase methane production because acetate feeds directly into the methanogenesis (Bouallagui et al. 2005).
Complex cell wall structure present in algal and plant feedstocks limits the hydrolysis step in anaerobic digestion because it makes organic matter difficult to break down (Passos et al. 2015). If nutrients cannot be broken down through hydrolysis, nutrients used to fuel methanogenesis are not sufficiently produced. Pretreatment of organic matter before undergoing anaerobic digestion can increase cell wall disruption, biomass solubilization, and ultimately methane production (Passos et al. 2014). Previous studies have shown that thermal and hydrothermal pretreatments increase methane yield considerably (Passos et al. 2015, Passos et al 2014, Cesaro and Belgiorno 2014). I therefore tested the effect of hydrothermal, or autoclaved, pretreatment of the algal feedstock on methane production.

Methods

To study the effects of treatments on anaerobic digestion over time, I established a series of incubations that I destructively harvested at 4 time points to test for gas and dissolved organic carbon (DOC) concentrations. The incubations housed a mixture of digester fluid (containing methanogens) and treated algal fluid. I tested four treatments: a control, glucose, acetate, and autoclave treatment. Each treatment had two replicate tubes at 4 time points and one replicate at the final time point, for a total of 36 incubations.

I used 18 x 150mm Chemglass Anaerobic Tubes with 20mm blue butyl rubber stoppers and aluminum seals to provide a gas-tight environment for incubation. This environment kept the fluid anaerobic and contained the methane and CO₂ produced during incubations. Each 27 mL vial contained 20mL liquid and 7 mL headspace. For the 20mL of liquid, I added 10mL digester fluid and 10mL algal fluid. To obtain the algae and digester fluid to fill the 36 tubes, I removed 500 mL of fluid from the A2M algal reactor and 500 mL of fluid from the A2M digester using syringes. I separated 100mL algal fluid for each of the four treatments. I left 100 mL of control algal fluid untouched. For both glucose and acetate treatments, I added sufficient glucose or acetate, respectively, to 100 mL of algal fluid to produce 100μM glucose (or acetate) in the final 1:1 mix of digester and algal fluid. I added a lower concentration of glucose and acetate to the incubation tubes than originally added to the digester in September. I did this in order to try to observe a peak and then a drop off in production over the experiment’s short time frame of 3 weeks. Finally, I subjected the fourth batch of 100mL algal fluid to pressurized, thermal pretreatment by autoclaving at 15 psi and 121°C for an hour, then cooling the solution. I
filled the incubation tubes in a glove bag continuously sparged with nitrogen (N$_2$) gas to ensure anaerobic conditions; all digester and algal fluids were continuously bubbled with N$_2$ gas until tubes were filled and capped. Filled tubes were placed into a dark container in the same Conviron PGR15 environmental chamber (Conviron, Winnipeg, Canada) that currently houses the A2M bioreactor system. Incubation tubes remained in the container in complete darkness at 25 °C until their designated harvest time.

At 2 days, 6 days, 13 days, and 20 days after the start of the incubations, I destructively harvested 2 tubes from each treatment to analyze dissolved inorganic carbon (DIC), methane, and dissolved organic carbon (DOC). In order to assess trends after the fourth time point, I also harvested one remaining tube from each treatment at 26 days. To test for DIC (CO$_2$) and methane production, I acidified liquid samples and ran the gaseous products through a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan). Briefly, for each vial, I measured the pressure of the headspace. Then, I injected 5 mL of N$_2$ gas into the headspace so that I could easily remove 5 mL of liquid into a syringe equipped with a luer lock port. Through this port, I then added 15 mL of CO$_2$-free air to the syringe, and then acidified the liquid sample with 2 μL 1 N H$_2$SO$_4$. After shaking the syringe for 2 minutes to ensure all CO$_2$ produced from carbonate and bicarbonate species moved to the headspace, I discarded the 5 mL of liquid and injected the remaining 15 mL gas headspace into the gas chromatograph (GC) to measure CO$_2$ and methane concentrations. I determined the ppmv of CO$_2$ and methane in this 15mL volume of syringe gas by comparing the measured peak heights to standard curves relating heights to ppmv CO$_2$ and methane.

I then calculated the moles of CO$_2$ and moles of methane in the syringe headspace using the ideal gas law. I assumed standard temperature and pressure and complete partitioning of all CO$_2$ and methane from the liquid into the headspace:

$$n = \frac{PV}{RT} = \frac{ppm \times 15}{10^6 \times 1000} \times \frac{1}{(0.08205)(293)}$$

Equation (1)

I multiplied the resulting amounts of CO$_2$ (in various forms of DIC and CO$_2$) and methane derived from 5mL of incubation fluid by four in order to calculate the total amounts present in the original 20mL present in the incubation tubes.
To calculate the amount of CO$_2$ and methane in the vial headspace, I used Henry’s law, which relates gas species concentration in a solution with the partial pressure of that gas in the space above the liquid. My tubes contained 20mL of fluid and 7mL of headspace. Assuming equilibrium conditions, I used Henry’s law and the Henry’s law constant of 1418.55 uM/atm to calculate the amount of methane present in the vial headspace (Sander 2015):

$$P_{\text{methane}}(\text{atm}) = \frac{\text{Concentration of methane in solution}}{\text{Henry’s law coefficient for methane}} = \frac{\mu\text{mol/L}}{(\mu\text{mol/L})/\text{atm}}$$ \hspace{1cm} \text{Equation (2)}

I used the ideal gas law to calculate moles of methane in the original headspace using the calculated partial pressure of methane. Total moles of methane in each vial, found by summing the moles of methane in the headspace and moles of methane in the vial liquid, was plotted through time.

Measurements for total CO$_2$ required a similar calculation dependent on pH and partitioning. CO$_2$ dissolves into water and exists in several forms such as bicarbonate and carbonate. The partitioning of CO$_2$ depends on the pH of the liquid and the partitioning of carbon (C) in DIC into dissolved CO$_2$ vs. bicarbonate and carbonate. By knowing the pH of the solutions, we know what proportion of CO$_2$ produced by microbes exists as H$_2$CO$_3$ – a gas in equilibrium with CO$_2$ in the vial headspace. I calculated the concentration of H$_2$CO$_3$ based on the pH and total moles C in DIC I obtained from GC measurements. I used this concentration and a Henry’s law constant of 34,450 uM/atm to calculate the partial pressure of CO$_2$ in the vial headspace (Sander 2015). Using the ideal gas law, I then calculated moles of CO$_2$ in the vial headspace. I added the calculated amounts of CO$_2$ in the vial headspace and the vial liquid to obtain the total amount of CO$_2$ (partitioned in various forms) in the tubes. I then plotted these values over time.

After gas analysis, I filtered a portion of the remaining liquid into DOC vials using ashed Whatman® 25mm glass fiber filters. I diluted each 8mL liquid sample with distilled water and preserved with 5 uL per mL sample with 85% H$_3$PO$_4$. I dried the filters, then packed them in Costech® tin capsules. I then analyzed each filter for carbon and nitrogen content using a Thermo Scientific™ FLASH 2000 CHN Analyzer. DOC vials remained refrigerated until samples could be run for DOC. I ran these samples through an Aurora 1030 TOC Analyzer to receive DOC concentrations.
I performed 2-way ANOVA tests using RStudio to test for treatment and day effects on CO₂ production, methane production, and DOC concentration. I used Tukey’s HSD posthoc tests to determine which particular treatments or days differed significantly from one another.

**Results**

The glucose, acetate, autoclave treatments did not result in methane production significantly greater than the control (Figure 3). However, there was a very significant treatment, day, and treatment:day interaction effect (Table 1). A Tukey’s HSD posthoc test revealed that control and autoclave treatments did not significantly differ (p=0.876). The last time point, at which only one replicate was sampled, suggests a trend that methane production in the autoclave treatment surpasses the control. The data at this time point was not statistically significant due to a lack of replicates. The acetate and glucose treatments did not differ (p=0.999) but produced significantly less methane than the control and autoclaved treatments (p<0.05).

Extremely significant treatment and day effects existed among incubation CO₂ production (Table 2). CO₂ concentration in the autoclaved treatment was significantly higher than that found in the control, glucose, or acetate treatments (for autoclave vs. each other treatment, p=0.0000000). CO₂ production increased for all four treatments between days 2 and 6, but quickly plateaued and remained constant for the remainder of the experiment (Figure 2). It was not until the last time point when I harvested one incubation tube per treatment that CO₂ production was shown to increase once again. CO₂ concentrations increased in a similar trend among all four treatments (Figure 2). The control glucose, and acetate treatments did not differ in CO₂ concentrations (p>0.75).

DOC measurements indicated very significant treatment and day effects (Table 3). DOC was significantly higher in the autoclaved treatment than the other three treatments (p<0.0004) (Figure 4).

Neither treatment nor day had significant effects on the C:N content in the tubes.

**Discussion**

Contrary to my predictions, none of the three altered treatments stimulated higher methane production. Additionally, the glucose and acetate treatments inhibited methane production rather than stimulating it.
It is possible that tubes contained substantial amounts of volatile fatty acids (VFAs) such as acetic acid, propionic acid, and butyric acid created during the process of anaerobic digestion. The presence of VFAs can decrease pH to levels toxic to methanogens and other anaerobic organisms. Propionic acid alone can inhibit methanogen growth and methane production without causing severe decreases in pH (Barredo and Evison 1991, Wang et al. 2009). At all time points, the pH values measured in the tubes were within the range 6.50 to 6.90, which is below the ideal pH of 7.6 for methanogenesis (Steinhaus et al. 2007). Since the pH values in the tubes were lower than the ideal pH, the acidity in the tubes may account for lower methane yield. However, since the pH of each tube remained consistent over the course of the experiment, I ruled out changes in pH as a probable cause for lowered methane yield in any one treatment. However, perhaps the glucose and acetate treatments caused an increase in toxic substances such as propionic acid that inhibited methanogenesis without decreasing pH.

The plateau in CO₂ production after 6 days, while methane concentrations continued to increase, is also puzzling. By testing pH of vial liquid after acidification with 1 N H₂SO₄, I ruled out the possibility that the buffering capacity of the 5mL solution was so strong as to prevent acidification of liquid below pH 4 by 2μL of acid. Below pH 4, all DIC moves into the headspace as CO₂ as required for the GC quantification. The addition of 1 N H₂SO₄ lowered pH to below pH 2, and addition of extra acid did not drive more CO₂ out of solution (data not shown). A possible explanation for the observed plateau in CO₂ concentration after day 5 is diauxic growth within the incubation tubes. For the first few days, acetoclastic methanogenesis dominantly occurs. Then, hydrogenotrophic methanogenesis might have started to occur and consume hydrogen and CO₂ (Bouallagui et al. 2005). This could explain why CO₂ production plateaued while methane production continued to increase over the course of the incubations.

DOC in the autoclaved treatment was significantly higher than in the other treatments. I attribute this to how hydrothermal pretreatment disrupts cells. This disruption of cell structure during the process of autoclaving likely releases the inner organic contents of the algal cells into the incubation fluid. This increased dissolved organic content could then also contribute to the higher CO₂ production in autoclave treatment because there is more organic material for decomposers to digest and break down.

While treatment and pretreatment methods in the incubations did not significantly stimulate methane production, perhaps a more equal mix of reactor fluids (like the 1:1 ratio in
the tubes) could increase methane yield in the A2M digester itself. This would be a favorable option in that it would avoid the nutrient addition or physical pretreatment of algae that require external or high-energy input (Passos et al. 2015). In the future, it would be interesting to test how increasing the ratio of these two reactor fluids in the anaerobic digester could affect methane production through adjustment of reactor volumes or flow rate. In order to support a higher fluid mix ratio, perhaps the flow rate between the two microcosms could increase greatly. While this technology needs to be perfected, the idea of a closed-system algae-to-methane bioreactor is a novel and promising idea that could one day provide a clean, alternative source of energy.

Acknowledgements
I would like to thank my mentors, Zoe Cardon and Joe Vallino, for their time, guidance, and invaluable input. I am deeply grateful to Suzanne Thomas for her indispensable help and company during this project. I’d also like to thank Rich McHorney and Jane Tucker for their assistance with laboratory machinery.

Literature Cited


Figures and Tables

FIGURES
Figure 1. Methane concentration in A2M digester over time. 1mM acetate was added on day 111 and 1mM glucose was added on day 117.

Figure 2. Plot of methane present (both in liquid and headspace) in incubation tubes over time. Last time point had only 1 replicate.

Figure 3. Plot of carbon (distributed in bicarbonate species and CO₂ gas) present in incubation tubes over time. Last time point had only 1 replicate.

Figure 4. Dissolved organic carbon (DOC) in incubation tubes at 4 harvest points. Last time point had only 1 replicate.

TABLES
Table 1. Results of two-way ANOVA assessing effect of treatment, day, and their interaction on production of CO₂ in incubation tubes.

Table 2. Results of two-way ANOVA assessing effect of treatment, day, and their interaction on production of methane in the incubation tubes.

Table 3. ANOVA statistical analysis of treatment, day, and interaction effects of incubation tube DOC concentration.
Figure 1.
Figure 2.
Figure 3.

CO₂ production over time

Treatment effect:
p = 6.4 × 10⁻¹⁰
Figure 4.
Table 1. Results of two-way ANOVA assessing effect of treatment, day, and their interaction on production of methane in the incubation tubes.

<table>
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<th>Methane</th>
<th>Df</th>
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<th>Mean Sq.</th>
<th>F value</th>
<th>Pr(&gt;F)</th>
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<td>232.57</td>
<td>77.52</td>
<td>7.332</td>
<td>0.0026**</td>
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<td>Day</td>
<td>3</td>
<td>2548.4</td>
<td>849.47</td>
<td>80.337</td>
<td>7.32e-10***</td>
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<td>Treatment:Day</td>
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<td>416.11</td>
<td>46.23</td>
<td>4.373</td>
<td>0.0051**</td>
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**Table 2.** Results of two-way ANOVA assessing effect of treatment, day, and their interaction on production of CO$_2$ in incubation tubes.

<table>
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<th>CO$_2$</th>
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Table 3. ANOVA statistical analysis of treatment, day, and interaction effects of incubation vial DOC concentration.

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<th>Mean Sq.</th>
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