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Increased methane production in cyanobacteria and methanogenic microbe co-cultures

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Abstract

A novel light-to-bioenergy system produced 3.5 times the baseline methane output using a co-culture of cyanobacteria (*Oscillatoria* sp.) and a methanogenic microbial community. Analysis of micronutrients in the system during the growth phase indicated that cobalt, iron, nickel and zinc were not appreciably consumed. The stable consumption and return of macronutrients calcium and magnesium were also observed. Essential macronutrients nitrogen, in the form of nitrate, and phosphorus showed no cycling during the growth phase and were depleted at rates of 0.35 mg/L/day and 0.40 µg/L/day, respectively. Biofilm formation increased the resilience of biomass to bacterial degradation in an anaerobic digester, as shown by viability assays of cyanobacterial biofilms in the co-culture.

Keywords: Bioenergy; Biofilms; Nutrient Cycling; Methanogenesis

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1. Introduction

The coupling of microalgae cultivation and anaerobic digestion represents a biogenic pathway to methane generation, which has the potential to produce large amounts of renewable fuel for gas-fired electricity generation plants (Frigon et al., 2013; Santos-Ballardo et al., 2016). A wide range of research is currently underway to resolve many issues that exist in bioenergy production. Many bioenergy systems involve high cost, energy-intensive processes. Despite significant research directed towards the development of feasible and efficient production of biofuels, many of these remain unsustainable (Ho et al., 2014; Liew et al., 2014). Anaerobic digestion is a promising bioenergy conversion technology (Batstone and Virdis, 2014; Sawatdeenarunat et al., 2015), and microalgae is a popular biomass feedstock (Maity et al., 2014; Ullah et al., 2015) that together have the potential to address these problems.

A self-contained biogas system that combined these processes and enabled cycling of effluents was demonstrated by Golueke and Oswald in 1959 to be sustainable (Golueke and Oswald, 1959). Since then, there has been increased interest and research on the sustainable production of biofuels from closed-loop systems that involve microalgal growth and anaerobic digestion (Alcántara et al., 2013; Ras et al., 2011). Recently, Jin and Borthwick introduced an aerobic decomposition stage into the system for more efficient nutrient recycling (Jin and Borthwick, 2016). However, bottlenecks in these multi-stage systems can be caused by degradation resistant microalgal feedstocks that require higher retention times (Kwietniewska and Tys, 2014). Thus, this study proposes and investigates a novel system, which is constructed by co-culturing cyanobacteria with methanogenic microorganisms. This system also aims to remove

inefficiencies in previous work that involved biomass transfer between separate vessels by allowing several processes to be performed simultaneously within a single bioreactor.

The co-culture attempts to produce methane by emulating the metabolic processes of phytoplankton, bacteria and methanogens, which have been proven to coexist in natural environments (Briée et al., 2007). A study on methane generation from hydrogen produced by cyanobacterial nitrogen fixation demonstrates the short-term viability of an intimate co-culture of cyanobacteria and methanogenic archaea (Berg et al., 2014). However, there is little information on the potential for sustained methane generation from biomass degradation in a co-culture of these microorganisms.

The need to harvest and concentrate microalgae for optimal anaerobic digestion is an obstacle to achieving financial viability in many algal digestion systems (Ward et al., 2014). A range of harvesting techniques have been attempted with varying success, cost and environmental impacts (Uduman et al., 2010). Biofilm formation is a naturally occurring method of concentration and has been demonstrated to be a promising solution to this problem (Berner et al., 2015; Gross et al., 2013). Microalgal biofilm cultivation has successfully produced high lipid contents for biofuel production (Shen et al., 2016), but there are still many unknowns regarding the behaviour of biofilms in anaerobic digestion systems.

Many studies have highlighted nutrient recycling as a critical step in reducing fertiliser requirements and achieving feasible microalgal bioenergy systems (Bennion et al., 2015; Peccia et al., 2013; Quinn and Davis, 2015; Silva et al., 2015). Nitrogen and phosphorus are important and are commonly regarded as limiting nutrients to microalgae cultivation (Elser et al., 2007). Numerous studies have also confirmed the

importance of calcium, cobalt, iron, magnesium, nickel and zinc for photosynthesising organisms such as cyanobacteria (Brand and Becker, 1984; Daday et al., 1988; Farhat et al., 2016; Nies, 1999) and methanogens (Gonzalez-Gil et al., 1999; Murray and van den Berg, 1981; Scherer et al., 1983). There is extensive research on the recycling of wastewater as a nutrient source for microalgae (Cai et al., 2013; Lowrey et al., 2015). However, there is no information on the potential for nutrient cycling within a methane-producing co-culture similar to the system proposed in this study.

Here, the possibility of establishing a co-culture system within a single vessel for methane generation has been explored. This study addresses the lack of data on the anaerobic digestion of biofilms and nutrient cycling in a bioreactor capable of sustained methane production, with the goal of producing a novel, passive light-in biogas-out energy system. Methane output was monitored to determine the methanogenesis potential of the system. The health and sustainability of the co-culture were assessed by examining changes in select macronutrient and micronutrient concentrations. The potential of biofilm use as a feedstock for methane production was also explored by assessing the resilience of biofilms in the system.

2. Material and methods

2.1. Bioreactor Systems

Four experiments were performed using 500 mL glass bioreactors (Schott). To each reactor, approximately 250 mL of in-house modified black sea media was added, and varying amounts of sand and pumice to provide a suitable environment for the growth of microorganisms. Microorganisms introduced to the reactors included planktic cyanobacteria, cyanobacterial biofilms (*Oscillatoria* sp.) and a digestion community of

bacteria and archaea, locally sourced from near-surface coastal marine sediments (beach sand). Planktic and biofilm cyanobacteria were cultured in f media. Each experiment was constructed in quadruplicate (details of the experiments are listed in Table 1). The headspace of each reactor was flushed with CO₂ and N₂ was added at 100 psi. Reactors were maintained at ambient temperatures and Bioreactors A, C and D were kept in partial darkness by covering the lower half of the vessel with aluminium foil.

The modified black sea media consisted of locally sourced surface seawater with added KBr (0.09 g/L), KCl (0.66 g/L), CaCl₂·2(H₂O) (0.30 g/L) and MgCl₂·6(H₂O) (3.10 g/L). This solution was autoclaved and cooled under N₂ purge of the headspace and 30 mL of 1M NaHCO₃ was added. 1 mL of trace element solution and 1 mL of an in-house vitamin solution was also added. The trace element solution consisted of CuSO₄·5(H₂O) (19.6 mg/L), ZnSO₄·7(H₂O) (44.0 mg/L), CoCl₂·6(H₂O) (22.0 mg/L), MnCl₂·4(H₂O) (360.0 mg/L) and Na₂MoO₄·2(H₂O) (12.6 mg/L). The vitamin solution consisted of Biotin (1 g/L), Vitamin B₁₂ (1 g/L) and Thiamine HCL (200 mg/L).

The f media consisted of NaNO₃ (150 mg/L), Na₂SiO₃·5H₂O (22.7 mg/L), Ferric Citrate (9.0 mg/L), Citric Acid (9.0 mg/L) and NaH₂PO₄·2H₂O (11.3 mg/L). 1 mL of trace element solution and 1 mL of vitamin solution were also added.

2.2. Methane Yields

Methane concentrations in the bioreactor headspace gases were monitored using a gas chromatography-mass spectrometry (GC-MS) system. Analysis was performed on a Focus GC connected to a DSQ II single quadrupole mass spectrometer (Thermo Fisher Scientific). 500 µL samples of headspace gases were made using a 500 µL headspace gas tight syringe (SGE) and manually injected for each analysis. Gas contents were separated using an RT-QPLOT capillary column (30 m × 0.32 mm ID; Restek). Only

two from four of each bioreactor system was analysed for each time point to minimise disturbance to the system headspace.

2.3. Biofilm Viability

Viability assays of cyanobacterial biofilms were performed using fluorescence microscopy. Biofilm samples were physically extracted from the reactors and incubated with 1 μ M SYTOX Green nucleic acid stain (Thermo Fisher Scientific) in the dark for 15 min, following the method used by Rychtecký *et al.* (2014) (Rychtecký *et al.*, 2014). A Leica TCS SP5 microscope was used equipped with a 100x NA1.4 oil objective, and the samples were excited with the 488 nm Argon-ion and 633 nm Helium-Neon lasers. Emitted fluorescence was detected using HyD detectors over wavelength ranges of 500-600 nm (green fluorescence) and 650-750 nm (red fluorescence).

2.4. Nutrient Analysis

Water samples were extracted from all four experimental quadruplicates for nutrient analysis. Nitrate was quantified using flow injection analysis (FIA) on a Lachat FIA, and calcium and magnesium were measured using inductively coupled plasma optical emission spectrometry (ICPOES) using a Perkin Elmer Optima DV7300 ICPOES. All other elements (cobalt, iron, nickel, phosphorus and zinc) were quantified using inductively coupled plasma mass spectrometry (ICPMS) with a Perkin Elmer NexION 300D ICPMS.

3. Results and Discussion

3.1. Methane Yields

Headspace methane concentrations (Figure 1) gave an indication of the level of methanogen activity in each bioreactor.

All methanogen inoculated reactors displayed the expected methane production, with the control systems all showing no activity over the observation period. The light supplied to Bioreactor B effectively inhibited the activity of the photosensitive methanogenic archaea, resulting in no methane output. Similarly, methane concentrations were constant in Bioreactor D due to the lack of a suitable feedstock for the bacterial and archaeal community. Bioreactor A, which was not inoculated with methanogenic archaea, had a methane concentration that was lower than the bioreactors in series B and D. The higher concentrations in reactors B and D are explained as the base-level methane produced, due to remaining trace methanol and cyanobacteria feedstock in the methanogen inoculant.

Bioreactor C showed a methane yield higher than control systems by a factor of 3.5, with additional methane production appearing to cease 17 days after the addition of biomass feedstock. The decrease in methane at 28 days can be attributed to biological variability between samples as different pairs of samples were analysed over the observation period. The gradual increase in methane concentration from days 7 to 17 confirm the potential for sustained biogas production from the co-culture. However, further methane generation may be hindered by the resilience of the cyanobacterial biofilms to bacterial degradation. The formation of biofilms gives microorganisms greater protection as individual cells are encased within an extracellular matrix, which has been shown to protect against environmental stresses and external attack (Branda et al., 2005; Flemming and Wingender, 2010). This likely prevented the degradation community from readily accessing any biomass beyond the surface of the biofilm. In comparison, the planktic cyanobacteria introduced to the system had less protection from bacterial attack and were more readily removed to facilitate the production of

methane. In addition, the resilient cyanobacteria may be performing photosynthesis and producing oxygen. The oxygen generated during photosynthesis may also hinder methane production by the anaerobic methanogens if there are insufficient aerobic microorganisms present to scavenge dissolved oxygen.

Qualitative observations and viability assays of the cyanobacterial biofilms, discussed in Section 3.2, showed that healthy biofilms were present in the reactors. These observations supported the supposition that the biofilm structure provided increased resilience against bacterial degradation. Planktic microalgae have also been observed to have inherent resistance against degradation, due to the high lipid content of the cells (Foree and McCarty, 1970), but longer hydraulic retention times (HRTs) were effective at increasing organic compound removal by the anaerobic digesters (Ras et al., 2011).

3.2. Biofilm Viability

Fluorescence microscopy was used to perform viability assays of biofilms introduced into Bioreactors A, B and C. The viability assays at 14 and 31 days after introduction to the bioreactors showed minimal degradation relative to positive controls (completely dead biofilm). The images revealed that the population of viable cells in biofilms extracted from the bioreactors were comparable to the number found in the live sample viability assay. These results were consistent with the resilience of the biomass to bacterial attack. SYTOX Green predominantly stains nucleic acids and dead cyanobacteria were indicated by green fluorescence in the centre of the red-autofluorescent cells. However, green fluorescence that was not associated with the strands of cyanobacteria was also observed in the biofilms. This was most apparent in the images taken of biofilms after 31 days in the bioreactor. Nucleic acid components of

the biofilm matrix (Flemming and Wingender, 2010) could have bound the stain and resulted in this extracellular signal. However, these assays suggested that the biofilms were relatively healthy and had remained resilient against bacterial attack, given the relatively harsh conditions expected to be inside of the bioreactors. This may result in a lag in methane output as the microbial decomposers require more time to overcome the resistance of the biofilms. In addition, the reduced surface area offered by biofilms in comparison to planktic cyanobacteria may also be a factor causing slower degradation.

Environmental stresses cause microorganisms to form biofilms, which has previously been observed when planktic algae were introduced into anaerobic digesters and fixed biomass was found (Ras et al., 2011). A similar phenomenon was noted in this work, with planktic cyanobacteria adhering to the glass walls of most reactors and beginning to form biofilms. Most of these adherent cells transitioned from deep green to brown in colour (chlorotic), one indicator of the death of cyanobacteria, but remained attached to the reactor wall. In contrast, the complete digestion of any planktic cells in the reactors was indicated by the transition of the system media from green to clear and colourless over the observation period, even when agitated. These newly formed biofilms did not appear to completely survive bacterial attack.

It is thought that bacterial breakdown of biomass during anaerobic digestion was more difficult when an extracellular matrix forms in biofilms. This theory is supported by the comparison between these adherent cyanobacterial cells and their planktic counterparts.

The resilience of the extracellular matrix of the biofilm is likely to have contributed to the observed health of the cyanobacterial biofilms. However, this could also be attributed to the possible nitrogen fixing properties of cyanobacteria from this group

(Oscillatoriales). As discussed in section 3.3.1, the nitrate in the bioreactor systems was depleted over time. This depletion, as well as the anaerobic conditions of the reactor, could induce nitrogen fixation. Thus, the cyanobacteria were provided with the essential macronutrient, and the health of the biofilms was maintained.

3.3. Nutrients Analysis

3.3.1. Macronutrients

The concentrations of the macronutrients nitrogen (in the form of nitrate), phosphorus, calcium and magnesium in the reactors are presented in Figure 2.

The availability of nitrogen in the systems was monitored by quantifying the concentration of nitrate over time. Nitrate was introduced into Bioreactors A, B and C as a component of the f media in which planktic cyanobacteria had been growing. Excepting Bioreactor D, which contained no nitrate, this vital macronutrient was depleted at similar rates for all reactors. Depletion of nitrate was found to be at a rate of approximately 0.35 mg/L/day or 0.52 $\mu\text{M/hr}$. This is comparable to estimated nitrate uptake rates for the related cyanobacterium *Phormidium laminosum* of 0.25 – 0.5 $\mu\text{M/hr}$ (Hu et al., 2000). These results showed that nitrate was not cycled over this time span, or that a sustainable equilibrium requires higher initial nitrate concentrations. However, it did not provide conclusive evidence that nitrogen is not being recycled, just that nitrogen was not returned in measurable quantities to the media for biomass uptake as nitrate.

The decomposition of algae returns nitrogen to the system, most commonly in the form of ammonia (Koster and Lettinga, 1984). The release of ammonia has been observed in the anaerobic digestion of algae (Samson and LeDuy, 1983; Yen and Brune,

2007). This suggests the potential of nitrogen cycling occurring in the reactors, facilitated by ammonia release.

Phosphorus is an essential macronutrient for microorganisms and occurs in nature as organic and inorganic phosphate (Fulekar, 2010). Phosphorus concentration was observed to fall in the systems from day 3 to day 21 and stabilise at zero. However, significant fluctuations were visible in the data, some of which may be due to systemic uncertainties. This is possibly the result of small variations in the acidification of the aqueous media during sample preparation before ICP measurements. The fluctuation was particularly pronounced at 14 days. An absence of significant fluctuations in other major nutrients, such as nitrate, provides further evidence for this being a systemic error. Regardless, all reactors displayed eventual depletion of the macronutrient, with depletion rates of around 0.40 $\mu\text{g/L/day}$. Complete consumption of phosphorus in Bioreactor D indicated insufficient initial concentrations to sustain the growth of the bacterial community. This also suggests that phosphorus levels were too low for the co-culture of multiple microorganism communities to reach a stable equilibrium. The depletion of phosphorus in Bioreactors A, B and C could also be explained by the phosphorus storage properties of cyanobacteria (Thompson et al., 1994). Phosphorus is accumulated and stored in microalgae as polyphosphates (Eixler et al., 2006), which could account for the low concentrations of the nutrient in the system media.

The concentrations of calcium were relatively constant in all systems except for Bioreactor B. The large calcium concentration increase and decrease observed in Bioreactor B, but not Bioreactors A and D, suggested that calcium was returned from cyanobacteria feedstock that had undergone bacterial breakdown. Since bacterial breakdown of cyanobacteria was also expected in Bioreactor C, the absence of

fluctuations for this reactor substantiated the occurrence of very efficient calcium recycling in the bioreactor. Similarly, constant magnesium concentrations observed were also a potential indicator of effective cycling. However, in cyanobacteria *Anacystis nidulans* growth was only limited by magnesium below 5 μ M (Webb et al., 2003), and the reactors contained comparatively high magnesium concentrations. Thus, the constant trend across all reactors was most likely due to the insignificant uptake of magnesium. Calcium and magnesium are necessary for photosynthesis (Brand and Becker, 1984; Farhat et al., 2016) and are an important component in methanogens (Scherer et al., 1983). However, high levels of these macronutrients in seawater suggests this element is not quickly depleted in nature. The results of this study also indicate that the depletion of magnesium should not be expected in the short term within this system.

3.3.2. Micronutrients

The concentrations of cobalt, iron, nickel and zinc in the bioreactors are shown in Figure 3. Cobalt, iron and nickel have been shown to stimulate methanogen metabolism (Gonzalez-Gil et al., 1999; Murray and van den Berg, 1981), while iron is important for photosynthesis and nickel is necessary for nitrogen fixation in cyanobacteria (Daday et al., 1988; Marsh et al., 1963). Zinc is needed in many enzymatic processes carried out by microorganisms (Scherer et al., 1983). The importance of these trace elements for all microorganisms in the system suggests that these nutrients should be transferred between organisms within the bioreactors. The results showed fluctuations in micronutrient concentrations, which indicated that the return of these micronutrients could be expected in the complete system. Comparison between bioreactor experiments

showed that micronutrient return to the system was dependent on the presence of degradation bacteria.

Fluctuations in cobalt, nickel and zinc concentrations suggested cycling of these nutrients in Bioreactor A, which only contained cyanobacteria. This can be explained as the result of biofilm development observed in the bioreactors, which involves cell death (Webb et al., 2003). Under nutrient-limited conditions, cells in a biofilm will also undergo autolysis to release nutrients (Allocati et al., 2015). Cyanobacteria are also capable of sequestering or exporting zinc to the periplasm (Blindauer, 2008). This could explain the observed increases in zinc concentration if cells were broken apart during inoculation or sampling with a syringe needle.

A large increase in iron concentration was observed at 35 days for all bioreactors. A similarly large increase in iron concentration was observed when comparing iron levels in black sea media with methanogen cultures used for this work. This suggests the large increase in iron is not a systemic error. The increase in iron could be explained as the return of this nutrient by the breakdown of microorganisms. Studies have shown that methanogens are capable of reducing iron oxides in marine sediments (Sivan et al., 2016), which could also contribute to the increase in iron content in the media observed at longer times and nutrient depleted conditions. The micronutrient concentration fluctuations, particularly in Bioreactors B and C, provided evidence that these elements were being consumed and returned to the system at varying rates.

Cobalt, zinc and nickel levels appeared to be trending towards a steady state over the observation period. Assuming that the consumption of these nutrients follows an exponential decay trend, the data suggests cobalt and nickel levels tend towards zero

but stabilise at a small, non-zero value. The zinc concentration data for Bioreactor D suggested that zinc levels would stabilise at over 200 µg/L. However, zinc levels in the other reactors would eventually deplete with an average rate of 0.03 µg/L/day.

3.4. Summary of Further Work

The results of this study raise various questions that require further investigation. The nutrient uptake and return data in this study warrants further investigation for a larger co-culture and at shorter time intervals. In particular, confirmation of the nutrient data could be achieved with a comparable study carried out over a longer period of time. Further work with a stronger microbial co-culture is also needed to monitor microorganism populations with minimal disturbance to the operation of the system. Most importantly, essential macronutrients such as nitrogen and phosphorus must reach a non-zero steady state in the system to allow the sustainable operation of a methane-producing co-culture.

4. Conclusions

This is the first reported instance of sustained methanogenesis from cyanobacteria and methanogens co-cultured in a single vessel. Methane was produced for at least 10 days with yields 3.5 times higher than from the controls. Cyanobacterial biofilms showed increased resilience to bacterial degradation when compared to planktic cyanobacteria, although longer retention times could allow the bacteria to overcome this resilience.

Cobalt, nickel and zinc exponential decay suggest the availability of these nutrients will not be an issue in the short term. However, nitrate and phosphorus were depleted, highlighting the need for further investigation to achieve a long-term sustainable bioenergy system.

E-supplementary data for this work, which contains more specifics on the methods used and data analysis details, can be found in the e-version of this paper online.

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Figure Captions

Figure 1: Bioreactor Methane Yields. Appreciable methane concentrations approximately 3.5 times that of control levels were observed for Bioreactor C, with a lack of methanogen activity in all other control reactors. Methanogen activity continued in Bioreactor C for approximately 17 days before ceasing, likely as a result of the resilience of cyanobacterial biofilms to bacterial digestion. A decrease in methane around 28 days is the result of biological variability in the samples as two different sets of samples were analysed during this period. Methane yields were normalised against the average methane concentration of Bioreactor D. Error bars represent standard deviation between identical samples or were estimated using a sigmoidal fit to individual datasets to obtain average error for each point.

Figure 2: Bioreactor Macronutrient Concentrations. Monitoring of the major nutrients required by the system, nitrogen (in the form of nitrate) and phosphorus showed initial depletion at rates of 0.35 mg/L/day and 0.40 µg/L/day respectively (assuming exponential decay), highlighting an area for further study optimising in-situ nitrogen fixation. Large calcium concentration fluctuations in Bioreactor B but not Bioreactors A and D suggested return of calcium from the degradation of cyanobacteria. Subsequently, relatively constant calcium levels in Bioreactor C indicated the potential existence of very efficient recycling in the bioreactors. The same constant trends in magnesium were likely a result of insignificant nutrient uptake rates. A systemic analysis error in the phosphorus measurements may have occurred at day 14. The expected trend for phosphorus concentration is shown with a dotted line. Estimates for the initial values are indicated.

Figure 3: Bioreactor Micronutrient Concentrations. Analysis of micronutrients

demonstrated cycling of necessary trace elements in the reactors. Cobalt, zinc and nickel concentrations appeared to trend towards a steady state. Exponential decay curves suggest that depletion of these three trace metals will not pose an issue in the short term. Estimates for the initial values are shown.

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Table 1: Description of different bioreactor systems.

Bioreactor	Description	Contents
A – Cyanobacterial control	A cyanobacteria control. Containing only cyanobacteria (planktonic and biofilm).	150 mL sand 5 g pumice 250 mL black sea media 3 g cyanobacterial biofilm 70 mL planktonic cyanobacteria
B – Bacterial community control	A bacterial community control. Containing cyanobacteria (planktonic and biofilm) and the digestion community, exposed to light to inhibit the growth of the photosensitive methanogens.	250 mL black sea media 3 g cyanobacterial biofilm 70 mL planktonic cyanobacteria 30 mL methanogens
C – Complete system	The complete system. Containing cyanobacteria (planktonic and biofilm) and the digestion community, stored in the dark to ensure methanogen activity.	150 mL sand 5 g pumice 250 mL black sea media 3 g cyanobacterial biofilm 70 mL planktonic cyanobacteria 30 mL methanogens
D – Archaea control	An archaea control. Containing only the digestion community.	150 mL sand 5 g pumice 250 mL black sea media 30 mL methanogens

Highlights

- Methane yields achieved from the co-culture of cyanobacteria and methanogens.
- Nutrient cycling, excluding nitrogen and phosphorus, was observed.
- Cyanobacterial biofilms showed resilience against bacterial degradation.