Biomethane Production from Biodegradable Plastics

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BIOMETHANE PRODUCTION FROM BIODEGRADABLE PLASTICS

by

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ABSTRACT

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Organic polymer plastics are often short-lived commodities for single-use that result in landfill buildup and persistence in the environment. Plastic waste accumulation can cause ecological damage. Plastic production continues to outpace plastic waste management and perpetuates the growing epidemic of plastic pollution. More efficient handling of plastics would be beneficial.

One improvement involves biodegradable plastics (i.e., bioplastics), particularly polylactic acid (PLA) and polyhydroxyalkanoates (PHA), which can alleviate environmental concerns stemming from mismanagement. Yet, there are currently no bioplastic waste management strategies scalable to handle the millions of pounds of bioplastics that enter the waste stream. Therefore, new bioplastic resource recovery options were investigated through anaerobic co-digestion, a potential solution that can take advantage of existing digesters to convert bioplastic to biogas containing methane for renewable energy.

Bioplastics biodegrade, but their potential to completely biodegrade on a time-scale compatible with current anaerobic digestion technologies is largely unknown. Accordingly, base-catalyzed thermal pretreatments were investigated to increase biodegradation rates. Batch experiments revealed pretreatments at 55 °C, pH 12 for PHAs and 90 °C regardless of pH for PLA produced the greatest increase in subsequent bioconversion to methane. Polyhydroxybutyrate (PHB) showed the highest rate of methane recovery and was selected for high-rate anaerobic co-digestion investigations simulating full-scale anaerobic digestion at municipal water resource recovery facilities. Synthetic municipal primary solids were co-digested with untreated or pretreated PHB at a 15 d retention time and resulted in 79-93% and 84-98% bioplastic conversion to methane, respectively, corresponding to a 5% additional increase when pretreated. Microbial communities analyzed via Illumina sequencing showed archaea were unchanged in response to PHB co-digestion, whereas the bacterial community changed, with increased relative abundance of *Kosmotoga*, *Deferribacter*, *Geobacter*, and *Ruminococcus*. Therefore, these taxa may be important for PHB biodegradation.

The results of the current study suggest anaerobic co-digestion at municipal water resource recovery facilities is a feasible waste management option for PHB bioplastics, which may help to alleviate challenges associated with contemporary single-use plastics. Near complete conversion of PHB bioplastic to methane in just over two weeks signals a great compatibility with completely-stirred tank reactor co-digestion.

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DEDICATION

I would like to dedicate this thesis to my parents, Perry Benn and Cheryl Iwami-Benn, who have been a constant source of motivation and provision. Their guidance has taught me the value of hard work and persistence and has been vital in my life and academic pursuits. I would also like to thank the Gerndt brothers and Sabrina for their encouragement and emotional support throughout this project.

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1 INTRODUCTION

1.1 Motivation

Most plastic waste is non-biodegradable and causes environmental problems. A potential solution relies on new, biodegradable plastics. A cradle-to-cradle scenario involves anaerobic digesters in which bioplastic may be converted to biomethane (Figure 1.1). Bioplastics tested include polyhydroxybutyrate (PHB) and polylactic acid (PLA). We propose to develop a new pretreatment and anaerobic digestion process to convert bioplastics to biomethane for renewable energy. Processing and pretreatments required for rapid anaerobic digestion of bioplastics, their biomethane yields, and microbial community compositions have not been previously determined to the author's knowledge.

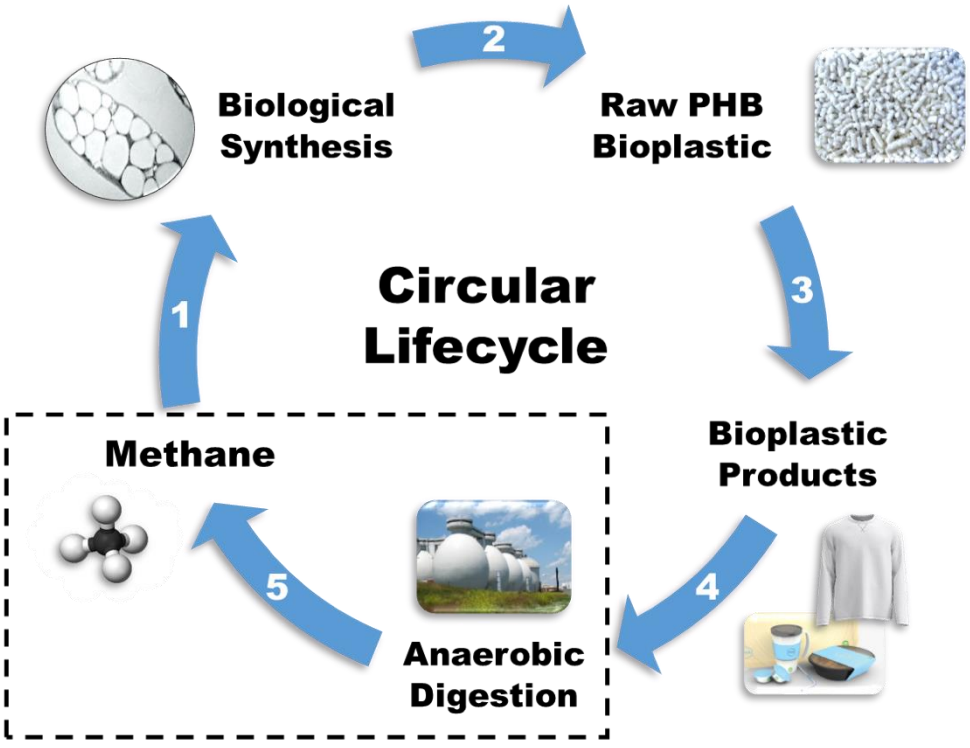

Figure 1.1 Circular lifecycle for PHB bioplastics and methane with a focus on step 5, biologically converting post-consumer bioplastics back to methane through anaerobic digestion (inspired by Rostkowski et al., 2012).

1.2 Hypotheses & Objectives

The following three hypotheses with associated research objectives were investigated:

(1) Base-catalyzed thermal pretreatment is necessary to render bioplastics amenable to digestion in the time scale of anaerobic digestion. Research objectives associated with this hypothesis were as follows:

- Develop bioplastic preprocessing protocol to establish uniform particle size
- Develop bioplastic liquid suspension base-catalyzed thermal pretreatment protocol for conditions at pH 7, 8, 10, and 12, temperatures at 35, 55, and 90 °C, and incubation time for 3, 24, and 48 hours.
- Screen each bioplastic temperature and incubation time pretreatment profiles with standardized biochemical methane potential (BMP) tests to identify optimum pretreatment profiles for increased biomethane yield.
- Screen pH conditions at the two most optimum pretreatment temperature profiles at all three incubation times with BMP tests to identify the optimum pretreatment conditions for increased biomethane yield. The most promising pretreatment profile of two PHB bioplastics are then used for bench-scale co-digestion investigations.

(2) Continuously fed, bench-scale co-digestion of pretreated PHB bioplastics will increase the biomethane yield compared to that of untreated PHB. Research objectives associated with this hypothesis were as follows:

- Prior to PHB co-digestion, quasi-steady state continuously fed anaerobic digesters treating a synthetic municipal primary sludge (SMPS) will establish consistent digester performance and microbial communities. This provides a baseline for comparison to PHB co-digestion.
- Following SMPS digestion, untreated and pretreated PHB was continuously co-digested until quasi-steady state to evaluate daily biomethane yield due to PHB and impact of pretreatment on the rate and extent of biomethane production.

(3) Feeding PHB as an anaerobic co-substrate will select microbial communities enriched for hydrolytic and fermentative bacteria, catalyzing the initial breakdown of polymeric substances, but have little impact on archaea. Research objectives associated with this hypothesis were as follows:

- Illumina sequencing of the highly conserved region of the 16S rRNA gene from pre-, transition, and post- PHB co-digestion phases will show relative abundance changes as co-digesters acclimate from SMPS substrate alone to addition of PHB.

1.3 References

Rostkowski, K.H., Criddle, C.S., Lepech, M.D., 2012. Cradle-to-gate life cycle assessment for a cradle-to-cradle cycle: Biogas-to-bioplastic (and back). Environ. Sci. Technol. https://doi.org/10.1021/es204541w

2 LITERATURE REVIEW

2.1 Anaerobic digestion and co-digestion of PHA

Anaerobic biodegradation studies of PHAs began in the 1980s when bioplastics began to be developed on an industrial scale for single-use commodity applications, like plastic beverage bottles (Holmes, 1985; Stieb and Schink, 1984). Previous, early studies laid the groundwork for future biodegradation studies by establishing fundamental knowledge and showing that PHAs are a naturally occurring microbial carbon storage polyester that is readily biodegradable. PHB and a related copolymer, poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), were studied with batch tests, pure culture plates, or enzymatic assays to determine their biodegradability over a defined period or until complete mineralization had taken place. Anaerobic degradability studies of PHAs primarily investigated inocula from anaerobic digesters at industrial or wastewater treatment plants (Budwill et al., 1992; Gartiser et al., 1998; Mergaert and Swings, 1996; Reischwitz et al., 1998; Yagi et al., 2014, 2013, 2009), various environmental sources, like pond sediments, rumen fluid, and spring water, (Budwill et al., 1996) as well as pure cultures (Janssen and Schink, 1993). Numerous PHA biodegradability studies utilized aerobic inocula from soils and other environmental sources (Brandi et al., 1995; Jendrossek et al., 1996; Mergaert et al., 1994, 1993; Schink et al., 1992), while one study named approximately 700 different microbial strains encompassing 59 different taxa that could degrade PHB (Mergaert and Swings, 1996).

Anaerobic biodegradation studies of bioplastics would resume, spurred by the emergence of a newly-available bioplastic called polylactic acid (PLA), for which usage has increased worldwide due to cost reductions from cheap feedstocks, technology

maturity, and economy of scale (Gross and Kalra, 2002; Muller et al., 2017). In 2018, it was estimated that 2.33 million tons of bioplastics were produced, 10.3% comprised PLA, nearly 240,000 tons, whereas PHAs accounted for 1.4%, approximately 32,600 tons (European Bioplastics, 2018). PLA bioplastic is different than PHA's in that the monomer, lactic acid, is produced through microbial fermentation and then polymerized through a series of industrial chemical processes (Lunt, 1998). PLA in its polymer form is not a microbial product and some anaerobic degradability tests have shown that it does not degrade as quickly nor yield as much biomethane compared to PHAs (Narancic et al., 2018). Yagi et al. (2009, 2013, 2014) found that PLA only began to degrade after 55 days at mesophilic temperatures to achieve up to 22-49% degradation within 277 days and required thermophilic conditions to reach degradation of 82-90% within 96 days. Criddle et al. (2014) similarly found that biogas generation from PLA was delayed approximately 35 days and biogas was nearly double after 120 days of incubation during thermophilic conditions compared to mesophilic conditions. Kolstad et al. (2012) and Vargas et al. (2009) also showed high rates of PLA degradation and biomethane yield during thermophilic digestion, 40-80% within 60 days. All other reports of anaerobic biodegradation of PLA at mesophilic temperatures revealed poor biomethane production or weight loss within 60-390 days of tests (Gartiser et al., 1998; Vargas et al., 2009; Endres and Siebert-Raths, 2011; Kolstad et al., 2012; Krause and Townsend, 2016; Narancic et al., 2018). However, PLA will degrade during industrial composting in which aerobic conditions cause high temperatures stemming from rapid biodegradation of organic matter.

Numerous studies have investigated PHAs as a component of municipal or industrial anaerobic digestion (Morse et al., 2011; Huda et al., 2013; Yagi et al., 2013, 2014; Soda et al., 2016; Wang et al., 2015, 2016, 2018; Narancic et al., 2018; Sethupathy and Sivashanmugam, 2018). A majority of these studies focused on batch anaerobic digestion tests that do not simulate operations that occur during typical continuous-fed digestion, and found that approximately 60 – 100% of PHAs were converted to biomethane. However, only one study briefly looked into continuously-fed co-digestion to analyze archaeal relative abundance, but this special case of intracellular PHAs within waste activated sludge organisms was studied and not the usable form of bioplastic (Wang et al., 2015). Wang et al. (2015) co-digested waste activated sludge containing PHA in the range of 21 (± 4) to 184 (± 16) mg PHA/g VSS (volatile suspended solids). The results of these studies indicate that even small amounts of PHA can rapidly increase biomethane production from anaerobic digestion.

The work described in this thesis focused on anaerobic digestion of exogenous PHA from commercial sources because its application is intended to degrade post-consumer PHAs and maximize biomethane production. Previous investigations have indicated that PHA can be anaerobically biodegraded and co-digested, whether the PHA was intracellular and at low OLR or exogenous PHA at much higher OLR.

2.2 Microbial community composition of anaerobic PHA degrading microbes

The understanding of biodiversity of PHA degrading microbes is developed for aerobic microbes, but anaerobic-correlated PHA degrading microbes have not been as

thoroughly studied (Mergaert and Swings, 1996). Studies conducted in the 1980s found that newly discovered anaerobic microbes could degrade hydroxybutyrate, the monomer comprising PHB (i.e., *Ilyobacter polytropus* (Stieb and Schink, 1984)) and a unique syntrophic bacterium, *Syntrophomonas wolfei* (McInerney et al., 1979; Wofford et al., 1986), that can grow when a H_2-utilizing microbe like a hydrogenotrophic methanogen is present. Two anaerobic microbes that can degrade PHB were found by pure culturing methods in the 1990s, *Ilyobacter delafieldii* (Janssen and Harfoot, 1990; Janssen and Schink, 1993) and a bacterium from *Clostridium* group I (strain LMG 16094) (Mergaert et al., 1996). Most of these early studies relied upon culturing techniques, gram staining, and microscopic analysis to characterize microbes.

Within the last few years, modern DNA sequencing technologies have allowed researchers to characterize more anaerobic microbes responsible for anaerobic PHA degradation. The report by Wang et al. (2018) was the only study found that utilized 16S rRNA gene Illumina sequencing technology for microbial community analysis of methanogenic PHA degrading batch tests. However, sample preparation was unconventional for anaerobic digesters, centrifuged digestate supernatant was filtered and membranes frozen, which may not have accurately reflected the microbial community. Bacterial orders *Cloacamonales*, *Thermotogales*, and two unidentified taxa were enriched, whereas archaea were not discussed.

Yagi et al. (2014) performed batch anaerobic digestion tests of PHB under mesophilic conditions with inoculum from an industrial anaerobic digester fed cow manure and vegetable waste and found eubacteria of an uncultured strain of *Clostridium* and *Arcobacter thereius* with low-level detection of archaeal strains including

Methanobacterium petrolearium, *Methanobacterium sp* (uncultured strain), and *Methanosaeta concilii* (Yagi et al., 2014). Yagi et al. (2013) similarly performed batch anaerobic digestion of PHB at thermophilic conditions and found eubacteria strains *Peptococcacea bacterium* Ri50, *Bacteriodes plebeius*, and *Catenibacterium mitsuokai* with no archaeal strains described. Yagi et al. (2013) also found different bacteria responsible for anaerobic digestion of three biopolymers together (PHB, PLA, and PCL – polycaprolactone), including *Bacillus infernus*, *Propioni bacterium sp*, and two uncultured strains; no mention of archaeal strains was made. The Yagi et al. (2013, 2014) studies utilized RNA extraction, reverse transcription-polymerase chain reaction amplification (RT-PCR), denaturing gradient gel electrophoresis (DGGE) profiles, and Sanger sequencing to detect and identify taxa based on their 16s rRNA sequence. Wang et al. (2015) operated semi-continuously fed anaerobic co-digesters to biodegrade WAS with intracellular PHA for 90 days. They investigated the relative abundance of archaea with a WAS feed containing low levels of PHA (21 mg PHA/g VSS) and high levels of PHA (184 mg PHA/g VSS) and found 34.5 ± 4.2% and 52.6 ± 5.7% archaeal abundance, respectively, based on 16s rRNA gene fluorescence in situ hybridization (FISH). Conversely, the Yagi et al. (2013) study described low detection of archaea, albeit their methods were not quantitative, whereas Wang et al. (2015) found very high abundance values of archaea, which may indicate inconclusive results and method bias, in terms of archaeal communities. The microbial communities and key microbial taxa involved in anaerobic digestion and co-digestion of PHAs, especially archaea, requires further investigation.

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3 PRETREATMENT and ANAEROBIC CO-DIGESTION of SELECTED PHB and PLA BIOPLASTICS

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3.1 Abstract

Conventional petroleum-derived plastics are recalcitrant to biodegradation and can be problematic as they accumulate in the environment. In contrast, it may be possible to add novel, biodegradable bioplastics to anaerobic digesters at municipal water resource recovery facilities along with primary sludge to produce more biomethane. In this study, thermal and chemical bioplastic pretreatments were first investigated to increase the rate and extent of anaerobic digestion. Subsequently, replicate, bench-scale anaerobic co-digesters fed synthetic primary sludge with and without PHB bioplastic were maintained for over 170 days. Two polyhydroxybutyrate (PHB), one poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and one polylactic acid (PLA) bioplastic were investigated. Biochemical methane potential (BMP) assays were performed using both untreated bioplastic as well as bioplastic pretreated at elevated temperature (35–90 °C) under alkaline conditions (8<pH<12) for 3–48 h. PHB and PLA pretreatment increased average BMP values to over 100%. Average PHB lag time before methane production started, decreased when pretreatment was performed. Bench-scale anaerobic co-digesters fed synthetic primary sludge with PHB bioplastic resulted in 80–98% conversion of two PHB bioplastics to biomethane and a 5% biomethane production increase compared to digesters receiving untreated PHB at the organic loadings employed (sludge OLR = 3.6 g COD per L of reactor volume per day [g COD/L_R-d]; bioplastic OLR = 0.75 g theoretical oxygen demand per L of reactor volume per day [ThOD/L_R-d]). Anaerobic digestion or co-digestion is a feasible management option for biodegradable plastics.

3.2 Introduction

Conventional plastics derived from petroleum are not biodegradable to a significant extent and result in accumulation of plastic waste in landfills or natural environments (Rostkowski et al., 2012). Conventional plastics accumulate most notably in oceans where they have been shown to disintegrate, forming microplastic particles that adsorb pollutants such as polychlorinated biphenyls (PCBs), pesticides, and phthalates (Andrady, 2011). Microplastic particles with sorbed pollutants can be consumed by marine organisms and enter the human food chain (Hammer et al., 2012; Mato et al., 2001).

To be considered biodegradable, bioplastics must exceed 90% carbon conversion to carbon dioxide during aerobic composting within 180 days (Brodhagen et al., 2017; Narancic et al., 2018). Polyhydroxybutyrate (PHB) bioplastic is biodegraded in aerobic and anaerobic engineered processes as well as natural environments; however anaerobic co-digestion of PHB for the express purpose of waste management and renewable energy has not been investigated (Abou-Zeid et al., 2004; Deroiné et al., 2014; Gómez and Michel, 2013; Volova et al., 2010). Budwill et al. (1996) reported that PHB is anaerobically biodegradable in various scenarios and suggested that municipal anaerobic sewage sludge digesters were suitable PHB degrading environment to generate biomethane. PHB was shown to anaerobically biodegrade over 90% in 10 days at mesophilic conditions, whereas polylactic acid (PLA) only biodegraded 7% in 90 days even though it is considered to be industrially compostable under aerobic thermophilic conditions (Yagi et al., 2014). Despite lesser biodegradability, PLA is more readily

available on the market today due to more efficient production at full scale (Gómez and Michel, 2013; Kolstad et al., 2012; Yagi et al., 2014, 2013).

To help mitigate the environmental concerns of conventional plastics, a more efficient coupling of bioplastic production and waste management should be developed (Gironi and Piemonte, 2011). According to cradle-to-gate lifecycle assessments (LCA), the biodegradable bioplastic PHB has potentially lower ecological impacts and global warming potential than conventional plastics if feedstocks are biobased and originate as by-products or wastes (Narodoslawsky et al., 2015). Other LCA researchers investigated PHB in a more holistic cradle-to-cradle scenario profiling an optimized process scheme with the assumption of complete biomethane recovery using anaerobic biodegradation and concluded that PHB was superior to conventional plastic in terms of global warming potential (Rostkowski et al., 2012). The assumption for complete biomethane recovery was described as an end of life option in which PHB was converted to biogas at an anaerobic digestion facility. Direct evidence supporting anaerobic digestion of bioplastics such as PHB to biomethane in a waste management scenario is limited. Anaerobic digestion feasibility is often assumed with results from anaerobic batch tests that may not accurately reflect operation of continuously fed digesters at quasi steady state.

Waste management and renewable energy generation from some biodegradable bioplastics could be achieved through anaerobic co-digestion using existing infrastructure and minimal process modification. With co-digestion, two or more feed materials, such as biodegradable plastic and municipal primary sludge, are fed to an anaerobic digester concomitantly. Co-digestion is implemented at some existing municipal water resource recovery facilities that often have excess capacity as well as boilers and electricity-

generating equipment that employ biomethane (Navaneethan et al., 2011). Onsite storage of bioplastics, like PHB, could supplement anaerobic digestion by providing a dense source of carbon that may be utilized to blend with other influent waste streams. PHB has a bulk theoretical oxygen demand (ThOD) of 2,200 g ThOD/L, whereas synthetic municipal primary sludge contains approximately 50 g COD/L. In addition, Stroot et al. (2001) suggested a C:N ratio for anaerobic digestion in the range of 20:1–30:1, but municipal sewage sludge for digestion was found to have C:N ratios ranging from 6:1 to 16:1, whereas the bioplastics contain C, but no N. Thus, co-digestion of bioplastics can increase C:N ratio to suggested values as well as result in increased biomethane production for renewable energy generation.

Bioplastics, like PHB and PLA encountered in the consumer market, are water insoluble, hydrophobic polyesters that can be hydrolyzed by water-soluble endogenous carboxylesterase enzymes secreted by microbes. Carboxylesterases, like PHA depolymerase or lipase, disrupt the ester linkages between bioplastic monomers and release them from bioplastic as water soluble molecules becoming bioavailable for microbial metabolism (Yoshie et al., 2002). An obligate anaerobic bacterium, *Ilyobacter polytropus*, was evaluated in pure culture and was found to ferment 3-hydroxybutyrate to acetate and butyrate (Stieb and Schink, 1984). In order to facilitate more rapid bioplastic transformation to biomethane on the time scale of municipal anaerobic digestion, the surface area could be increased through chemical and thermal processing and pretreatment. Abiotic hydrolysis or depolymerization of PHA bioplastics into monomeric constituents and intermediate breakdown products was demonstrated at a pH of 13 in 0.1

M sodium hydroxide aqueous solution at temperatures ranging from 60 to 70 °C and various incubation periods (Yu et al., 2005).

Over 70% abiotic degradation of PHB was demonstrated at 70 °C in 4 M sodium hydroxide after 4 h of treatment. Treatment of PHB in acidic solutions of sulfuric acid (0.05–2 M) at 70 °C for up to 14 h did not result in abiotic degradation (Yu et al., 2005). Near complete abiotic degradation of the copolymer PHBV was shown at 60 °C in 0.1 M sodium hydroxide after 18 h of treatment (Myung et al., 2014b). Thus, pretreatment in alkaline media at elevated temperatures induced polyester backbone hydrolysis resulting in release of water soluble breakdown products such as 3-hydroxybutyrate and crotonate, which have both been shown to support growth of strictly anaerobic microbes (Dörner and Schink, 1990; Janssen and Harfoot, 1990).

In this study, bioplastic thermal and chemical pretreatments were employed to increase the rate and extent of anaerobic digestion and co-digestion of commercially available PHB and PLA bioplastics. In order to elucidate the applicability of bioplastic pretreatments for anaerobic digestion and co-digestion, biochemical methane potential (BMP) assays were performed and methane yields were compared. Bench-scale anaerobic co-digestion of two PHB bioplastics, both pretreated and untreated, at quasi steady state with synthetic municipal primary sludge was then performed.

3.3 Materials and Methods

3.3.1 Bioplastics

Bioplastics tested include four PHB varieties including one poly(3-hydroxybutyrate-co-4-hydroxybutyrate) as well as one PLA (Table 3.1). ENMAT^{TM}

Y3000 powder and Mirel™ F1006 bioplastics were produced through fermentation of D-glucose. The PHB copolymer Mirel™ M2100 (4.4% 4-hydroxybutyrate) was produced through fermentation of D-glucose and 1, 4-butanediol. PHB produced by Mango Materials, Inc. was made from biomethane from an anaerobic digester. The PLA Ingeo™ 2003D was obtained from a commercial, cold drink cup and may have contained other proprietary additives not reported by the manufacturer; this bioplastic was produced by fermentation of corn-derived dextrose followed by polymerization.

Table 3.1 Summary of Bioplastics.

Bioplastic Name (Manufacturer)	**Abbreviation**	**Polymer**	**T_m [b], HDT [c] (°C)**	**Original Form**
ENMAT™ Y3000 (TianAn Biologic Materials Co.)	PHB1	PHB	176, NA	Powder
Mirel™ F1006 (Metabolix, Inc. & Telles LLC [a])	PHB2	PHB	165, 123	Pellet (thermo formed)
Methane-derived bioplastic (Mango Materials, Inc.)	PHB3	PHB	172, NA	Powder
Mirel™ M2100 (Metabolix, Inc. & Telles LLC [a])	PHB4	PHB [4.4% 4-HB]	169, NA	Pellet (extruded)
Ingeo™ 2003D (NatureWorks LLC)	PLA	PLA	145, 55	Cup (thermo formed)

[a] Manufacturing discontinued
[b] Melting temperature
[c] Heat distortion temperature provided by manufacturer

3.3.2 Bioplastics Processing and Pretreatment

Bioplastics were processed using methods similar to those reported by others (Witt et al., 2001; Yagi et al., 2013). Briefly, pelletized or thermoformed bioplastic samples were immersed in a liquid nitrogen bath for approximately 5 min to make them brittle and easier to grind, mechanically ground in a laboratory blender with a stainless steel canister (Waring 700G Commercial Blender), and sieved to less than 0.15 mm particle size. All bioplastics evaluated, apart from methane-derived PHB manufactured by Mango Materials, were commercially available at the time of testing. The Mango Materials plastic was obtained from the manufacturer as a prototype sample that was not yet commercially available. The commercially available bioplastics contain additives such as plasticizers and inks that may have influenced anaerobic digestion results.

Processed bioplastics were pretreated to increase surface area or initiate depolymerization to facilitate increased biomethane evolution during anaerobic digestion and co-digestion. Pretreatments were performed for each bioplastic using two methods. The first method involved only thermal pretreatment. This was done at 35, 55, and 90 °C for 3, 24, and 48 h at each temperature (9 different time-temperature conditions). The second method involved exposing the plastics to alkaline conditions with thermal pretreatment. Temperatures that resulted in the greatest 40-day BMP values using the first method were selected for subsequent alkaline-thermal testing at pH values of 8, 10, and 12 and incubation durations of 3, 24, and 48 h (3 pH values at 3 different holding times and 2 different temperatures yielded 18 different pretreatments for each bioplastic).

For pretreatment, a bioplastic suspension (25 g/L) in deionized water was placed into a 50 mL glass vial or 500 mL glass Erlenmeyer flask. The suspension was mixed with a magnetic stir bar and the pH was increased by sodium hydroxide addition. Thermal pretreatment was done in a water bath continuously mixed at 150 rpm on an orbital shaker (Stuart–Bibby Scientific SBS40 Shaking Water Bath). After thermal pretreatment, the slurry was allowed to cool to ambient temperature and the pH was adjusted to approximately 7 using hydrochloric acid. Pretreated, neutralized bioplastic suspensions were then dried with a laboratory air-blowdown evaporator to facilitate more accurate substrate distribution on a mass basis for anaerobic digestion evaluation.

Untreated and pretreated PHB2 samples were observed by scanning electron microscope (SEM) imaging to visualize the physical effect of thermal alkaline pretreatment. Surface morphology was captured via JEOL JSM-6510LV SEM imaging (JEOL Ltd., Akishima, Tokyo, Japan) under high vacuum at an accelerating voltage of 20 kV and magnifications of x500 and x5,000 PHB particles were mounted to SEM specimen mounts with carbon tape and sputter-coated with gold and palladium to a thickness of approximately 200 Å (20 nm).

3.3.3 Biochemical Methane Potential (BMP) Assays

BMP assays were employed to evaluate biomethane yields from untreated and pretreated bioplastics and reported at 40-day test duration unless otherwise noted at 15 or 60 days. BMP assays were performed in triplicate, as described elsewhere (Owen et al., 1979). Briefly, serum bottles (160 mL) were seeded with 50 mL of biomass and 5 mL of bioplastic slurry (25 g/L) containing either pretreated bioplastic, untreated bioplastic as

negative control (NC), 5 mL of de-ionized water as blank control (BC), or 5 mL of glucose solution (13 g/L) as positive control (PC). Serum bottles were capped with butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK) and crimped with aluminum seals. Setup was performed within a vinyl anaerobic glove box (Coy Laboratory Products, Grass Lake, MI) purged with nitrogen (N_2) gas and less than one percent hydrogen (H_2) gas. BMP assays were incubated (35 °C) with constant orbital mixing at 150 rpm (New Brunswick Scientific—Model C25KC, Edison, NJ). Serum bottle biogas volume was measured intermittently with wetted glass barrel syringes at ambient pressure and 35 °C, whereas serum bottle headspace methane concentration was determined by gas chromatography. All BMP values were calculated by subtracting the blank control biomethane production value from the BMP gross test value. Lag time was defined as the period between initiation of the BMP assay and the time when the biomethane production rate exceeded that of the blank control. Seed biomass was a mesophilic (35 °C) laboratory-maintained methanogenic, anaerobic biomass (15.5 ± 0.2 g/L total solids [TS], 7.1 ± 0.2 g/L volatile solids [VS]) fed dry milk substrate (3.5 g/LR-day) and basal nutrient media (Appendix 3, Table 3A) every day with a 15 day solids retention time (SRT) and continuous mixing. Biomass was stored for an average of approximately 1 week at 35 °C in 1 L amber glass jars with loose-fitted lids to allow for gas evolution prior to BMP analyses.

3.3.4 Anaerobic Co-digesters

Synthetic municipal wastewater sludge (SMWS) was digested alone or was co-digested with either untreated or pretreated PHB1 and PHB2 (see Table 3.1 for bioplastic

abbreviations) in duplicate anaerobic co-digesters (eight digesters total). Co-digesters were 2.5 L bench-scale, continuously stirred-tank reactors (CSTR) operated with a 15-day SRT and 15-day hydraulic residence time for 175 days. Conditions were maintained at 35.7 °C ± 2.1% and a constant mixing rate of 350 rpm using a magnetic stir bar. Co-digesters were seeded with mesophilic municipal anaerobic biomass (VS = 3.5%) from the South Shore Water Reclamation Facility (Oak Creek, WI). SMWS was composed of basal nutrient media, alkalinity (Appendix 3, Table 3A) and particulate substrate provided by ground dog food (1.21 ± 0.12 g COD/g dog food) sieved to less than 0.8 mm particle size having approximately 21% protein and 13% fat (Nutro Natural Choice, Franklin, TN, USA). Dry dog food provides a consistent, well-balanced substrate for consistent experimental digesters. SMWS was fed at an organic loading rate (OLR) of 3.6 g COD/LR-day, which was equivalent to 7.5 g dog food/day (Carey et al., 2016). The bioplastic OLR was 0.75 g theoretical oxygen demand (ThOD) per liter of reactor per day (ThOD/LR-d) which was approximately 20% of the COD OLR from SMWS alone. Control digesters were fed SMWS and untreated PHB bioplastic as a co-substrate.

SMWS was fed to all co-digesters without bioplastic from days 1 to 115; subsequently bioplastic was co-fed with SMWS from days 116 to 175. Digester performance was assessed by daily monitoring of temperature, pH, and biogas production as well as weekly biogas methane content, volatile fatty acids (VFA) concentrations, and solids analysis. Daily biogas volume produced was collected in gas sampling bags (Cole Parmer Kynar PVDF 20.3 L) and subsequently measured with a wet test meter (Precision Scientific). Bench scale anaerobic digestion lag time was defined as the period between day 115 when PHB co-digestion was initiated and the time when the rate of co-digester

biomethane production exceeded that of the digester fed SMWS alone. Quasi steady-state operation was defined as occurring after all digesters were operated under consistent conditions for at least three SRTs (i.e., 45 days) and biogas production rate values did not vary more than 10%.

3.3.5 Analyses

Biogas was analyzed for methane content by gas chromatography with thermal conductivity detection (GC-TCD) (GC System 7890A, Agilent Technologies, Irving, TX, USA) and data were reported at 35 °C and 1 atm. Total solids (TS), volatile solids (VS), and COD concentrations were measured by standard methods (APHA et al., 1999). VFA concentrations were determined by gas chromatography flame ionization detection (GC-FID) after samples were centrifuged, supernatant filtered through 0.45 µm syringe-tip filter, and acidified with phosphoric acid (Schauer-Gimenez et al., 2010). Since accurate bioplastic COD analysis was not achievable, the bioplastics ThOD values were calculated based on the bioplastic mass and molecular structure, with ratios of 1.67 g ThOD/g PHB and 1.33 g ThOD/g PLA. Bioplastics theoretical maximum methane production values (35 °C, 1 atm) were calculated using the Buswell Equation (Buswell and Mueller, 1952) and were 0.66 L CH_4/g PHB and 0.53 L CH_4/g PLA. Statistical analyses were performed in R Studio version 3.4.1. Normal distributions were not assumed, and significant differences among mean BMP values were determined using the non-parametric Mann-Whitney-Wilcoxon test with a confidence level of 0.95 and one-sided alternative hypothesis.

3.4 Results and Discussion

3.4.1 Bioplastic Pretreatment and BMP Assays

Pretreatment of PHB1 qualitatively resulted in visible surface erosion, increased porosity, and increased surface area compared to untreated (Figure 3.1). Increasing PHB surface area and porosity increases the available binding sites for biological enzymatic degradation and may therefore increase hydrolysis rates (Shang et al., 2012). Hydrolysis of recalcitrant substrates can be the rate-limiting step in methanogenesis, thus pretreatments that can facilitate increased rates of hydrolysis may increase the rate of methanogenesis (Venkiteshwaran et al., 2016). Thermal alkaline pretreatment of PHB and PLA bioplastics increased anaerobic biodegradability in terms of increased BMP values and reduced lag time compared to untreated controls as described below.

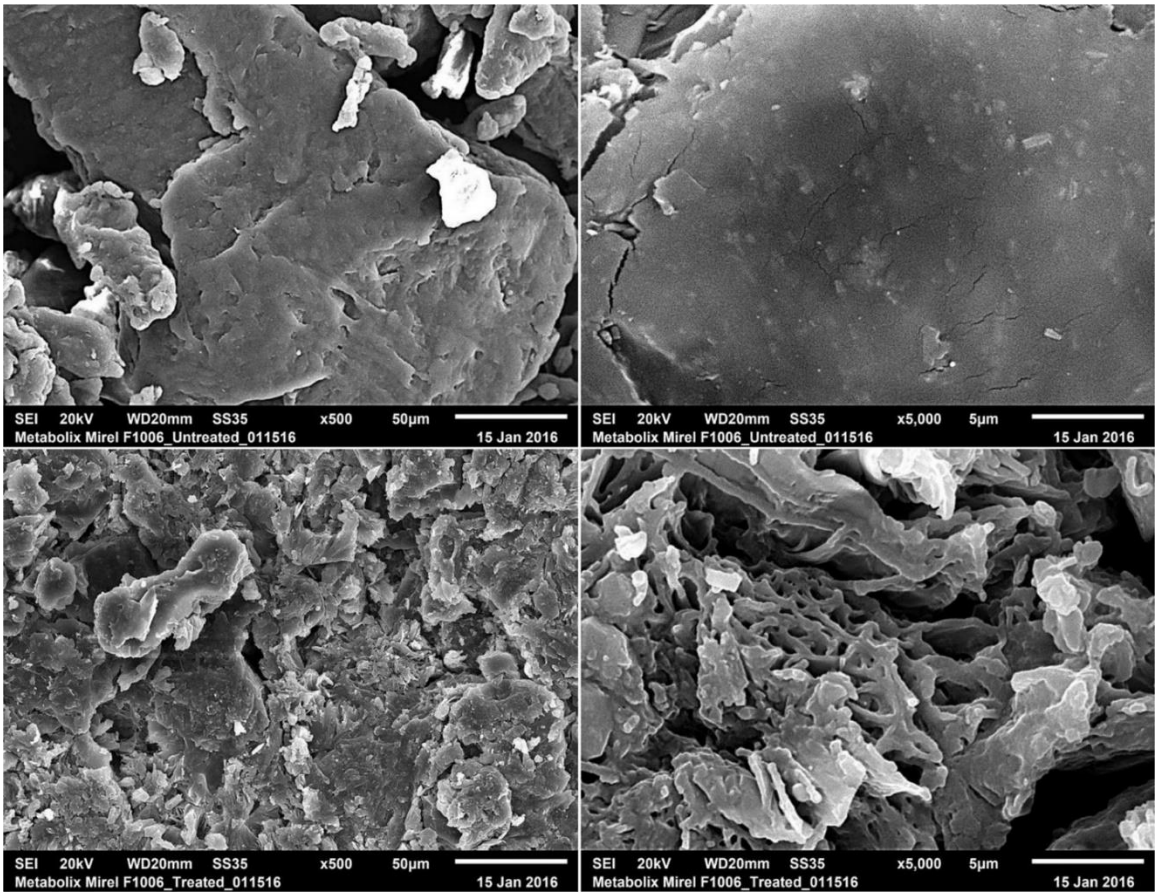

Figure 3.1 Scanning electron micrographs of untreated and pretreated PHB2 (Mirel^{TM} F1006) after processing. Untreated PHB2 at magnification x500 (top, left) and x5,000 (top, right). Pretreated PHB2 at 500x (bottom, left) and 5,000x (bottom, right), pretreatment conditions were 90 °C and pH 12 for 48 h.

BMP values and lag times resulting from 27 different pretreatment conditions (i.e., three temperatures at three pH values and three different contact times) for each bioplastic were determined and provided an initial assessment of biomethane production changes due to pretreatments for each bioplastic (see Appendix 3, Table 3B–3F). Percent conversion values for PHB and PLA to biomethane were calculated as the quotient of BMP value divided by the theoretical maximum methane production value determined from the bioplastic ThOD loading. Compared to untreated bioplastics, pretreated PHB

and PLA resulted in increased average BMP values. The pretreatment conditions resulting in the maximum increases in methane production are presented in Figure 3.2. Maximum percent conversion to biomethane for PHB was 101 ± 6% and 22 ± 6% for PLA after 40 days. Lag times of pretreated PHBs and PLA compared to untreated control digesters were reduced up to 60 and 98%, respectively.

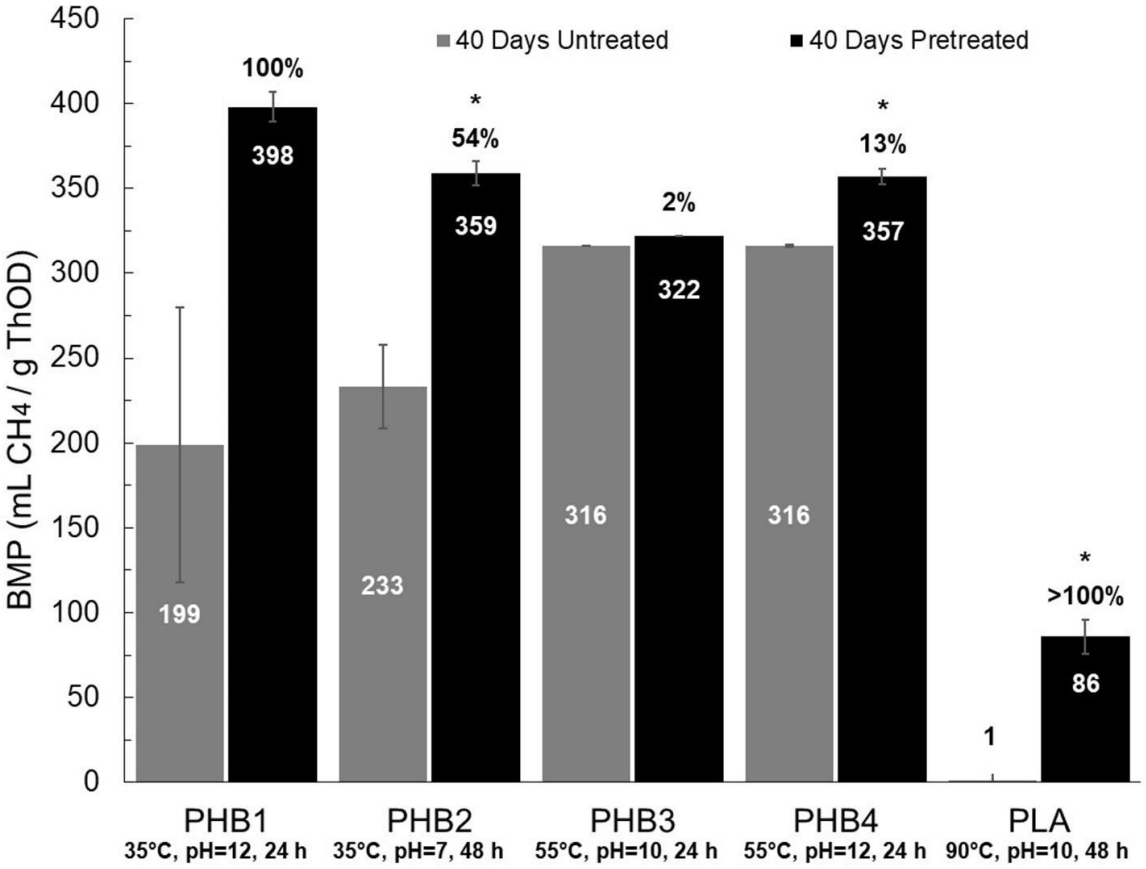

Figure 3.2 BMP values for untreated (gray) and pretreated (black) bioplastics under conditions resulting in the greatest biomethane increase. The specific conditions are written under each bar in the graph (temperature, pH, duration). BMP values, shown within each bar, with 40 days' duration are reported at 35 °C and ambient pressure. Percentages above black bars indicate relative increase from untreated to pretreated, with statistically significant differences at 95% confidence denoted by an asterisk (*). Error bars are relative standard deviation (n = 3); some error bars are small and not visible.

BMP values for pretreated PHBs averaged 360 ± 18 mL CH4/g ThOD (35 °C, 1 atm) representing 91 ± 4% conversion to biomethane, whereas untreated PHBs averaged 270 ± 71 mL CH4/g ThOD and converted 67 ± 19% to biomethane (Figure 3.2). An additional 20 days of BMP analysis yielded averages of 101 ± 4% and 76 ± 17% conversion for pretreated and untreated PHBs, respectively. Pretreatment led to statistically significant increased BMP values for PHB2 and PHB4, but not for PHB1 and PHB3 (see Appendix 3, Tables 3B–3E). Although the average BMP value of pretreated PHB1 increased by 100% compared to that of the untreated PHB1, the difference was not statistically significant due to high variance in the untreated BMP measurements (RSD ± 81%).

Methane-derived PHB3 exhibited rapid conversion to biomethane at 60 ± 1% after 15 days despite a negligible response to pretreatment. Other reports described untreated PHB conversion to biomethane at 39% in 5 days, 87% in 21 days, 92.5% in 22 days, and 100% in 98 days (Budwill et al., 1996, 1992; Yagi et al., 2014). Individual BMP results from each pretreated PHB vary, but the largest increase in BMP relative to untreated PHB were generally demonstrated at pretreatment conditions of 55 °C, pH value of 12, and 24 or 48 h pretreatment duration, which agrees with reports concluding that abiotic pretreatment of PHB at elevated temperature and pH produced degradation products (Yu et al., 2005).

Compared to untreated PLA, pretreatment of PLA resulted in the largest increase in BMP of the bioplastics studied (Appendix 3, Table 3F). Untreated PLA did not anaerobically degrade to biomethane, whereas pretreatment at 90 °C, pH value at or above 7 for 48 h significantly increased BMP to an average of 79 ± 8 mL CH4/g ThOD

and equivalent to as much as 22 ± 6% conversion to biomethane. Extending the BMP analysis another 20 days resulted in an additional 5% conversion to biomethane for PLA. Low PLA conversion to biomethane under mesophilic conditions has been reported by others. Kolstad et al. (2012) observed no biomethane evolution in mesophilic anaerobic digesters after 170 days, whereas others reported low conversion to biomethane from 12% at 77 days, 23% at 182 days, and up to 49% after 277 days (Yagi et al., 2014, 2009). In contrast, thermophilic anaerobic digestion of PLA was reported to yield higher rates of digestion with nearly 25% conversion to biomethane in 30 days and up to 75% in 75 days (Yagi et al., 2013). One study attempted pretreatment of PLA at 70 °C for 1 h with no pH control, but this resulted in less biomethane than untreated PLA (Endres and Siebert-Raths, 2011). Results from previous studies are in close accordance with the results herein. However, many of the previous investigations acclimated their seed inocula to enrich for bioplastic fermenting bacteria, whereas the work described herein did not. The BMPs reported herein are for unacclimated biomass that may result in longer lag time and lesser biomethane production within 40 days.

Thermal alkaline pretreatment of bioplastics generally resulted in reduced lag time compared to untreated bioplastics. Average lag time for untreated PHBs was greater than that for pretreated PHB. Untreated PLA did not yield biomethane after 60 days, but pretreated PLA demonstrated no detectable lag time (Figure 3.3). Lag times of untreated PHB3 were longer than those for pretreated PHB2 and highlighted that some commercial PHBs may not anaerobically degrade quickly, especially when using unacclimated biomass. The PHB3 was notable in that pretreatment did not result in a decreased lag time, whereas lag times for all other PHBs and PLA were reduced. In the case of PLA,

lag time was inversely correlated to pretreatment duration, with pretreatment times of 3, 24, and 48 h resulting in sequentially decreasing lag time of >3 weeks, 2 weeks, and no lag time, respectively (Figure 3.3E). Similarly, Yagi et al. (2009) reported a 55-day lag time for untreated PLA and others reported no anaerobic degradation for untreated PLA (Criddle et al., 2014; Kolstad et al., 2012). Yagi et al. (2014) suggested that mesophilic anaerobic microbial consortia may only have the ability to degrade low molecular weight PLA, and based on the BMP tests conducted here, it is possible that substantial methane production only occurred from low molecular weight PLA produced by thermal hydrolysis during pretreatments at 90 °C and 48 h. Longer pretreatment duration of PLA correlated to decreased lag time to the point when 48 h of pretreatment eliminated lag time altogether. PLA pretreatment at alkaline pH at 90 °C for durations longer than 48 h may result in increased BMP and potentially complete conversion to biomethane during anaerobic digestion.

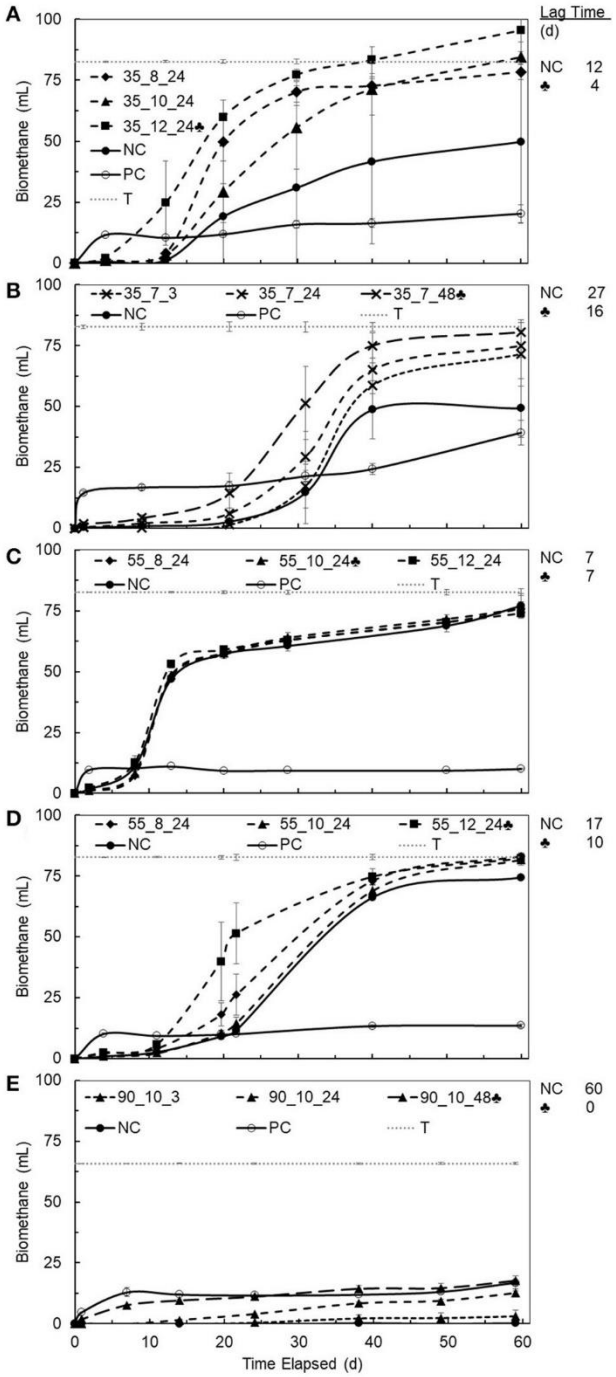

Figure 3.3 Average cumulative biomethane produced during BMP assays (n = 3, error bars and one standard deviation, 35 °C, ambient pressure) vs. time elapsed for PHB1 (A), PHB2 (B), PHB3 (C), PHB4 (D), PLA (E) after pretreatment. Conditions of pretreatment are denoted on each chart as temperature, °C _ pH _ incubation time, h. Dashed lines show incubation times and pH 8 (◆), pH 10 (▲), pH 12 (■), and highest biomethane production (♣). Solid lines show controls; negative control (NC •) was untreated bioplastic, positive control (PC ◦) was glucose, straight dotted line denotes theoretical maximum (T) biomethane production, and lag time shown to the right of each chart.

3.4.2 Bench Scale Co-digestion

Co-digestion of SMWS and PHB was feasible at bench scale as evidenced by efficient biotransformation to biomethane, while pH, temperature, VFAs, and VS removal remained stable (Table 3.2; Appendix 3, Figures 3A–3C). When bioplastics were co-digested, biomethane production increased 17% over that from digesting SMWS alone. Quasi steady state co-digestion of SMWS and PHB, after 45 days exhibited approximately 80–98% conversion of PHB to biomethane (Table 3.2). Calculations for conversion percentage of bioplastic to biomethane relied upon theoretical biomethane yield.

Table 3.2 Bench scale digestion and co-digestion meta data, (U, untreated; P, pretreated).

	SMWS Digestion				SMWS + PHB Co-Digestion			
	PHB1_U	**PHB1_P**	**PHB2_U**	**PHB2_P**	**PHB1_U**	**PHB1_P**	**PHB2_U**	**PHB2_P**
Biogas[a] (L/d)	5.7 ± 0.5	5.6 ± 0.5	5.6 ± 0.5	5.7 ± 0.5	7.0 ± 0.5	6.8 ± 0.7	6.7 ± 0.5	6.6 ± 0.3
pH	7.31 ± 0.02	7.29 ± 0.03	7.29 ± 0.02	7.29 ± 0.02	7.27 ± 0.05	7.24 ± 0.05	7.24 ± 0.04	7.25 ± 0.04
VFA (mg/L)	47 ± 3	51 ± 6	48 ± 5	46 ± 2	47 ± 4	47 ± 4	45 ± 2	45 ± 3
% VSR [b]	77 ± 1	76 ± 2	77 ± 1	76 ± 1	81 ± 1	78 ± 1	78 ± 1	78 ± 1
% VS	0.69 ± 0.02	0.72 ± 0.02	0.71 ± 0.02	0.73 ± 0.01	0.72 ± 0.02	0.83 ± 0.02	0.81 ± 0.01	0.81 ± 0.01
% CH_4	67 ± 3	67 ± 4	68 ± 4	67 ± 4	65 ± 0.4	64 ± 0.7	65 ± 0.4	66 ± 0.6

[a] Average and standard deviation values from duplicate digesters
[b] Percent volatile solids reduction (VSR) from feedstock to effluent

Average pH of digester effluent fed SMWS alone was 7.30 ± 0.02, while pH in all digesters dropped slightly after PHB was fed to the digesters. The pH difference was statistically significant during quasi steady state co-digestion with PHB at an average value 7.24 ± 0.02 (Appendix 3, Figure 3A). VFA concentrations of digester effluent expressed as acetic acid equivalents were 48 ± 4 mg/L and 46 ± 3 mg/L before and during co-digestion at quasi steady state for all digesters, respectively, and were not statistically different (Appendix 3, Figure 3B). The VS as a percent of TS in digester effluent deviated only 2% for all digesters and ranged between 57 and 59% (Appendix 3, Figure 3C).

The VS reduction (VSR) values increased for all digesters when PHB was co-digested and the average increased from as low as 75 ± 1% during SMWS digestion alone to as much as 81 ± 1% when bioplastic was co-digested. Solids initially increased in response to PHB addition but attained a quasi- steady state value after 15 days or one SRT. Average percent biomethane in biogas decreased from 2 to 3% when PHB was co-digested (Table 3.2), but the differences were not statistically significant.

In contrast to co-digestion of untreated PHB, co-digestion of pretreated PHB increased biomethane production by 5% and reduced lag time by approximately 4 days for both PHB1 and PHB2 (Figure 3.4). Lag time for bench scale co-digestion of PHB2 was 6 days for untreated and 3 days for pretreated bioplastic.

PHB co-digestion with synthetic primary sludge increased both the overall rate and extent of biomethane production compared to anaerobic digestion of synthetic primary sludge alone (Figure 3.4).

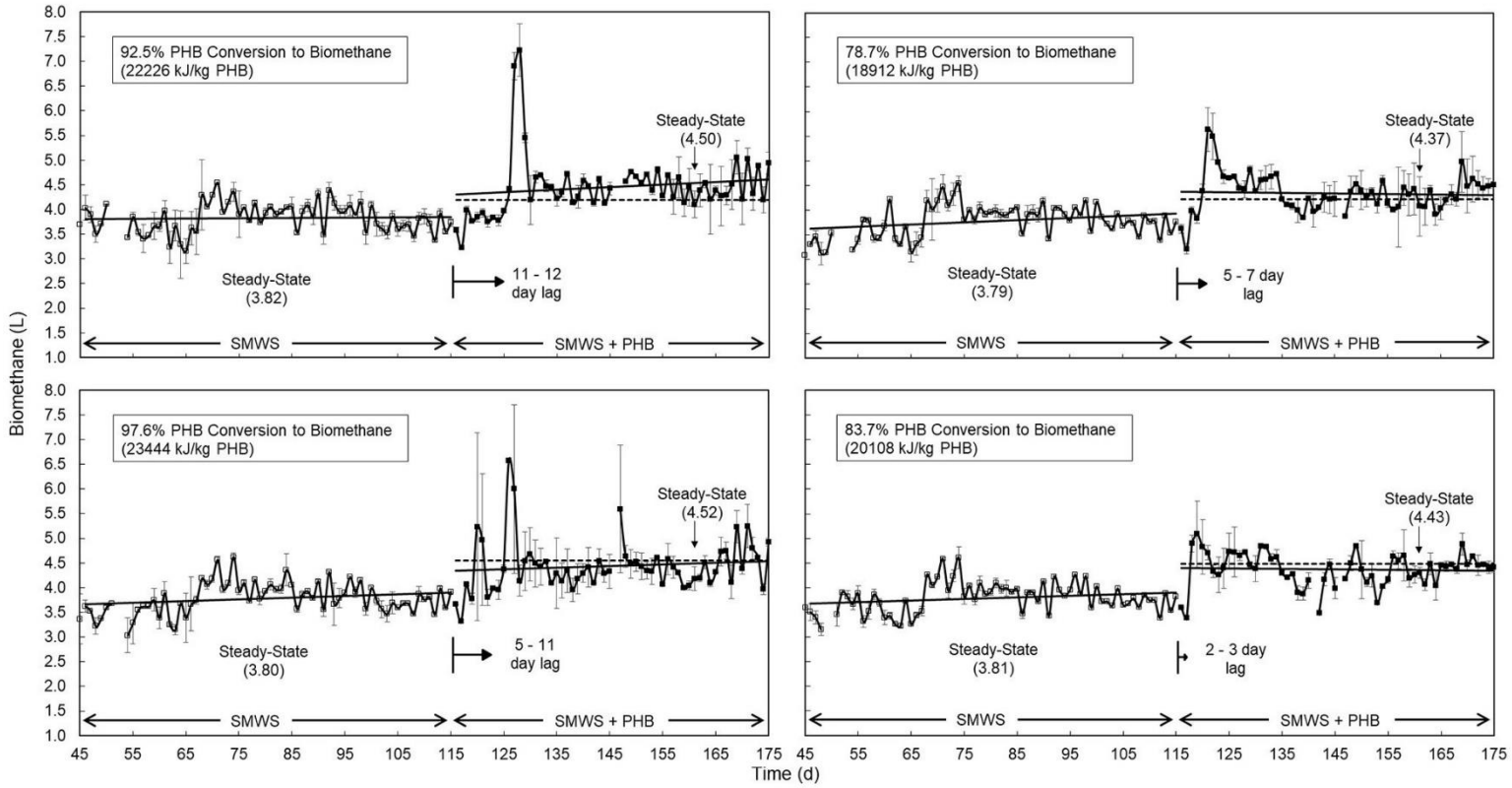

Figure 3.4 Daily biomethane production for continuously fed anaerobic digesters (n = 2, error bars show standard deviation) comparing (top, left) untreated PHB1, (bottom, left) pretreated PHB1 (treatment: 55 °C, pH = 12, 24 h) and (top, right) untreated PHB2, (bottom, right) pretreated PHB2 (55◦C, pH = 12, 48 h). Quasi steady-state was assumed after 45 days with average biomethane production (L/d) at quasi steady state presented in parentheses. Solid lines depict gas production rates before and after PHB co-digestion, dotted lines show theoretical co-digestion production based on 40 days BMPs. Solid arrows proportionately illustrate average lag period (d) between PHB addition and increased biomethane production. Steady state conversion of PHB to biomethane (%) and higher heating value of methane per kg PHB was based on an expected 21% increase in biomethane yield.

3.5 Conclusions

Biodegradable bioplastic can be co-digested under stable conditions at municipal water resource recovery facilities to generate renewable energy. Bioplastic pretreatment (≥55◦C, pH ≥ 10, ≥24 h) resulted in more rapid and complete anaerobic bioplastic co-digestion. With pretreatment, partial anaerobic digestion of PLA was accomplished. In addition, thermal alkaline bioplastic pretreatment reduced lag time before biomethane production occurred and increased bioplastic conversion to biomethane. Pretreatment of PHB bioplastic under quasi steady state co-digestion conditions resulted in approximately 5% greater biomethane production compared to untreated PHB. Bioplastic co-digestion at the loadings used increased biomethane production by 17%.

3.6 Acknowledgments

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4 METHANE YIELD and LAG CORRELATE with BACTERIAL COMMUNITY SHIFT FOLLOWING PHB BIOPLASTIC ANAEROBIC CO-DIGESTION

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4.1 Abstract

Past plastic management practices have resulted in pollution. An improved management scenario may involve adding used bioplastic to anaerobic digesters to increase methane for renewable energy. In this work, the effects of polyhydroxybutyrate (PHB) bioplastic anaerobic co-digestion with synthetic primary sludge on operation and microbial communities were investigated. Co-digesters treating sludge were co-fed 20% untreated or pretreated (55 °C, pH 12) PHB. Pretreatment resulted in shorter lag (5 d shorter) before methane production increased after co-digestion. At steady-state, co-digesters converted 86% and 91% of untreated and pretreated PHB to methane, respectively. Bacterial communities were different before and after bioplastic co-digestion, whereas no archaeal community change was observed. Relative abundance of 30 significant bacteria correlated with methane production and lag following PHB addition. No previously known PHB degraders were detected following PHB co-digestion. Microbial communities in anaerobic digesters treating synthetic primary sludge are capable of continuously co-digesting PHB to produce additional methane.

4.2 Introduction

Biodegradable polymer alternatives have been developed that could replace plastics derived from fossil fuel. However, most plastics are still currently produced from fossil fuels such as crude oil and are not bio-degradable in the timeframe of composting systems (Ali Shah et al., 2008; Geyer et al., 2017). The present lack of appropriate plastic waste management practices has resulted in as much as 79% of all plastic waste ever

generated, estimated at 6300 million metric tons as of 2015, to amass in the environment or landfills (Geyer et al., 2017). Conventional non-biodegradable plastics, namely single-use plastic packaging, can lead to contamination of land and aquatic environments. In addition, marine plastic pollution can cause ecological damage (Rochman et al., 2016). Plastic can fragment into smaller microplastic particles in the marine environment and act as a transport medium for harmful chemicals to enter the food chain (Mato et al., 2001).

Biodegradable plastic based on polyhydroxybutyrate (PHB) is one promising alternative to fossil-fuel-derived plastic (Emadian et al., 2017; Tokiwa et al., 2009; Tokiwa and Calabia, 2004). PHB bioplastics share similar properties with common thermoplastics such as polypropylene, and can often replace plastics produced from fossil fuel (Kalia et al., 2000; Verlinden et al., 2007). PHB is a form of polyhydroxyalkanoate (PHA) polyester produced by various heterotrophic microbes during stressed conditions, such as during carbon feast-famine regimes or nutrient limitation (Roohi et al., 2018; Verlinden et al., 2007). Industrially-relevant bacteria known to produce PHAs include, but are not limited to, *Alcaligenes latus*, *Cupriavidus necator*, and *Pseudomonas putida* (Kourmentza et al., 2017). The PHB granules stored by microbes internally can be extracted and purified to produce resin that may be used directly or may be copolymerized with other bioplastics to create application-specific blends (Kalia et al., 2000). Bioplastics derived from PHB are essentially completely biodegradable in aerobic and anaerobic engineered or natural environments (Getachew and Woldesenbet, 2016; Kalia et al., 2000).

PHB bioplastics can decrease economic and ecological impacts if the substrate used to produce them is biologically derived or originates from by-products or wastes

(Narodoslawsky et al., 2015). For example, methane derived from anaerobic digestion of waste can be used as a substrate to produce PHB by methanotrophic bacteria, specifically Type II Methanotrophs (class *Alphaproteobacteria*), under aerobic conditions (Pieja et al., 2011a, 2011b). Methane-derived PHB polymer is currently available from a commercial source (Mango Materials, Inc. Albany, CA, USA).

One plastic management scenario involves collecting and adding used PHB bioplastic to anaerobic digesters to increase methane production for renewable energy or for new bioplastic production. PHB contains no nitrogen and has a theoretical oxygen demand (ThOD) of 1.6 g ThOD/g PHB and yields 0.66 L CH_4/g PHB (35 °C) calculated from stoichiometric methane

$$C_4H_6O_2\ (PHB) + 1\frac{1}{2}\ H_2O \rightarrow 2\frac{1}{4} CH_4 + 1\frac{3}{4}\ CO_2 \qquad \textbf{(eq. 1)}$$

Continuous anaerobic digestion or co-digestion of PHB bioplastics to increase methane production has not been thoroughly investigated. In short-term, batch studies, the biochemical methane potential (BMP) values of five commercially available bioplastics including two PHB bioplastics produced from fermentation of D-glucose were determined and approximately 67% of the ThOD in raw PHB was converted to methane in 40 d under mesophilic conditions (Benn and Zitomer, 2018). Other studies have reported bioplastic digestion to methane with conversion efficiencies ranging from 39% in 5 d to 100% in 98 d under mesophilic conditions (Budwill et al., 1996; Yagi et al., 2014).

Initial hydrolysis of macromolecules such as PHB bioplastic is often the rate-limiting step for methane production. Pretreatment of PHB polymers using chemical and thermal processing could facilitate hydrolysis, resulting in more rapid bioplastic transformation to methane. Pretreatment under alkaline conditions at elevated temperatures has been shown to increase hydrolysis rates, resulting in release of water-soluble products such as 3-hydroxybutyrate and crotonate that can support growth of anaerobic microbes and support methanogenesis (Dörner and Schink, 1990; Janssen and Harfoot, 1990; Yu et al., 2005). Pretreatment at 55 °C and pH 12 for 24 or 48 h increased methane production from PHB from 67% to 91% (Benn and Zitomer, 2018).

The abundance of PHB degrading bacteria in anaerobic digester biomass also ostensibly affects the rate and extent of PHB conversion to methane. PHB bioplastics can be hydrolyzed by water soluble endogenous carboxylesterase, like PHA depolymerase or lipase, which disrupt the ester linkage between bioplastic monomers, releasing them as water soluble products available for microbial metabolism (Yoshie et al., 2002). A review

by Emadian et al. (2017) provided a list of isolated bacterial and fungal PHB degrading microorganisms in natural environments. The PHB degrading bacterial isolates were classified in the genera *Streptomyces*, *Burkholderia*, *Bacillus*, *Cupriavidus*, *Mycobacterium*, *Nocardiopsis*, *Pseudomonas*, *Enterobacter* and *Gracilibacillus* (Emadian et al., 2017). Most known PHB degrading bacteria have been isolated from compost or natural environments such as soil or river sediments contaminated by PHB, whereas there is no published work that has reported on the microbial community composition during anaerobic co-digestion of PHB bioplastics to our knowledge. Presence or enrichment of PHB degrading bacteria during anaerobic PHB co-digestion, and correlation between their abundance and digester performance could lead to strategies such as appropriate starting biomass selection or bioaugmentation to improve co-digester performance. In this study, bench scale, continuously fed, anaerobic co-digesters were used to convert two different untreated and pretreated PHB bioplastics as well as synthetic municipal primary sludge to biogas containing methane. Digester function and microbial community composition before and after initiation of PHB co-digestion were determined. Key taxa exhibiting significant relative abundance shifts after PHB was fed were correlated with observed digester methane yield and lag time.

4.3 Material and Methods

4.3.1 Bioplastic Processing and Pretreatment

Two different PHB bioplastics, ENMAT™ Y3000, TianAn Biologic Materials Co., China (PHB1), which is a fine powder, and Mirel™, Yield10 Bioscience, Inc., Woburn, MA, USA (PHB2), which is in pellet form, were employed. The two different

commercially available PHBs were used to discern if the source and form of PHB affects anaerobic bio-degradability. Bioplastic pellets were processed before anaerobic digestion using methods reported elsewhere (Witt et al., 2001; Yagi et al., 2013). Briefly, bioplastic was immersed in liquid nitrogen for 5 min to make it brittle and easier to grind in a laboratory blender (Waring 700G Commercial Blender). Ground bioplastic was sieved and the fraction with nominal particle size<0.15mm was anaerobically digested or pretreated before digestion.

Aliquots of processed bioplastic were pretreated in an effort to increase methane production. PHB1 was pretreated at 55 °C and pH 12 for 24 h, whereas PHB2 was pretreated at 55 °C, pH 12 for 48 h. These conditions were shown in previous work to result in maximum biochemical methane potential (BMP) increases compared to untreated controls (Benn and Zitomer, 2018).

4.3.2 Anaerobic Co-Digesters

Eight, 2.5 L anaerobic digesters with 2 L working volume were operated for 175 d. Digesters were continuously stirred-tank reactors (CSTRs), mixed at 350 rpm using a magnetic stir bar and operated with a 15-d hydraulic retention time (HRT) at 35 °C. Digesters were seeded with mesophilic anaerobic digester biomass (35 g VS/L) from a municipal water resource recovery facility (South Shore Water Reclamation Facility, Oak Creek, WI). During the pre-co-digestion period from days 1 to 115, all digesters were fed synthetic municipal primary sludge (SMPS) at an organic loading rate (OLR) of 3.6 g COD/L-d without bioplastic as a co-digestate. After the pre-co-digestion period, untreated or pretreated PHB bioplastics were co-fed with SMPS during the post-co-

digestion period from days 116 to 175. The PHB bioplastic OLR was 0.75 g COD/L-d, which was 20% of the SMPS OLR.

SMPS was composed of ground dog food (1.21 ± 0.12 g COD/g TS) sieved to <0.8 mm particle size having approximately 21% protein and 13% fat (Nutro Natural Choice, Franklin, TN, USA). The SMPS feed also contained basal nutrients and alkalinity in the following concentrations [mg/L]: NH_4Cl [400]; $MgSO_4*7H_2O$ [400]; KCl [400]; Na_2S*9H_2O [300]; $CaCl_2*2H_2O$ [50]; $(NH_4)_2HPO_4$ [80]; $FeCl_3*4H_2O$ [10]; $CoCl_2*6H_2O$ [1.0]; $ZnCl_2$ [1.0]; KI [10]; $(NaPO_3)_6$ [10]; the trace metal salts: $MnCl_2*4H_2O$, NH_4VO_3, $CuCl_2*2H_2O$, $AlCl_3*6H_2O$, $Na_2MoO_4*2H_2O$, H_3BO_3, $NaWO_4*2H_2O$, and Na_2SeO_3 [each at 0.5]; cysteine [10]; yeast extract [100] and $NaHCO_3$ [6000]. The SMPS composition was used in previous studies to simulate primary municipal sludge (Benn and Zitomer, 2018; Carey et al., 2016).

The eight digesters were divided into four sets of duplicates digesters. The first and second digester sets were fed SMPS with untreated and pretreated PHB1 bioplastic, respectively. The third and fourth digester sets were fed SMPS with untreated and pretreated PHB2 bioplastic, respectively. Lag time was defined as the period from day 115 (when PHB co-digestion was initiated) until the first day the methane production rate increased to the average methane production rate observed during the subsequent, post-co-digestion quasi steady-state period. Quasi steady-state was defined as the period after digester operation had been previously maintained under consistent conditions for at least three solids retention times (SRTs) (i.e., 45 d)

4.3.3 DNA Extraction and Illumina Sequencing Analyses

DNA was extracted and sequenced to monitor microbial community composition as described elsewhere (Carey et al., 2016; Venkiteshwaran et al., 2017). Digester effluent samples were collected for DNA extraction during the pre-co-digestion quasi steady-state period (days 91, 99 and 105), the transition period (days 121, 129 and 135) and the post-co-digestion quasi steady-state period (days 161, 168 and 175). DNA was extracted using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer protocol. Sequencing was performed using the Illumina MiSeq v3 300 base pair sequencing platform (Illumina, San Diego, CA). Universal primers 515F and 806R targeting the V4 variable region of 16S rRNA gene were used for PCR amplification. Raw unjoined sequence data were quality filtered (mean sequence quality score > 25). Barcodes and primers were removed from the sequences. Sequences with ambiguous base reads, fewer than 150 base pairs, and with homopolymer sequences exceeding 6 base pairs or longer were also removed. The de-noised sequences were then clustered into operational taxonomic units (OTUs) having 97% similarity. Each OTU was compiled into taxonomic “counts” and classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI.

4.3.4 Major, Minor and Significant OTUs

Major OTUs were defined as those with relative abundance values ≥0.1% in one or more samples, whereas minor OTUs were those with relative abundance <0.1% in all samples. Spearman's rank order correlation was performed using major OTUs to select

significant OTUs with relative abundance values in all digesters that correlated with average methane production rate, as described elsewhere (Venkiteshwaran et al., 2017). Spearman's rank order correlation was used as a measure of monotonic statistical dependence due to its robustness since it does not require underlying assumptions regarding the distribution frequency of variables (e.g., normal or uniformly distributed etc.) or the existence of a linear relationship between variables (Zuur et al., 2007). Only the quasi steady state pre- and post-co-digestion periods were considered for Spearman's order rank correlation. Major OTUs with relative abundance values that most positively related (i.e., Spearman's rank scores>0.75) and most negatively related (i.e., Spearman's rank scores less than −0.75) to methane production rates were categorized as significant OTUs.

4.3.5 Microbial Community Analyses

Richness (S), Shannon diversity (H) and evenness (E) indices were calculated using abundance data for all OTUs. Richness was calculated as the number of OTUs identified at the genus level. Shannon-Weaver diversity indices were determined as described by Briones et al. (2007). Evenness was calculated as described by Falk et al. (2009). Sequence reads were rarefied to even depth in R Studio with Phyloseq package using “rarefy_even_depth” (rngseed 3), 430 OTUs were removed due to zero reads present after random subsampling (Mcmurdie and Holmes, 2013). Analysis of similarities (ANOSIM) using Bray-Curtis dissimilarity was performed to compare the variation in taxa abundance values using the vegan package in R (Oksanen et al., 2016). ANOSIM analysis gives an ANOSIM statistic value (R) and a p value (significance of R). R values

close to 1 suggest high dissimilarity between groups, whereas values close to zero suggest no difference between groups. Spearman's rank order correlation was performed using Excel 2010 (Version 14.3.2 e Microsoft, USA) with the added statistical software package XLStat Pro 2014 (Addinsoft, USA). Non-metric dimensional scaling (NMDS) plots were produced using R Studio with Phyloseq package using “ordinate ()” with Bray-Curtis distances and constructed with “plot_ordination()”. Sample group ellipses at 95% confidence level were overlaid using “stat_ellipse()” from ggplot package (Fox and Weisberg, 2011; Mcmurdie and Holmes, 2013). Dual hierarchical clustering of pre and post-co-digestion samples was done in R Studio using “cor()” and “hclust” functions, and a heatmap was made in Excel 2010. Blast searching of representative sequences was conducted using default settings and excluding uncultured sequences on the browser-based blastn tool (https://blast.ncbi.nlm.nih.gov/) (Altschul et al., 1990).

4.3.6 Anaerobic digester Performance Analyses

Biogas was collected daily in gas sampling bags (Kynar PVDF 20.3 L, Cole Parmer, Vernon Hills, IL, USA) and the volume was measured with a wet test gas meter (Precision Scientific, Chicago, IL, USA). Biogas methane concentration was quantified by gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using a thermal conductivity detector. Volatile fatty acid (VFA) concentrations were measured by gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using a flame ionization detector. Volatile Solids (VS) and COD were determined by standard methods (APHA et al., 1999) and the pH was measured using a pH meter and probe (Orion 4 Star, Thermo, Waltham, MA, USA). Average, standard deviation,

variance and ANOVA calculations were performed using Excel 2010 (Version 14.3.2 e Microsoft, USA).

4.4 Results and Discussion

4.4.1 Anaerobic Co-Digester Function

During the pre-co-digestion quasi steady state period (Days 90 to 115) all digesters were operated similarly, and digester methane production rates were similar (p value>0.05, n=8), averaging 1.9 ± 0.02 L-CH_4/L-d (Figure 4.1; Appendix 4, Table 4A). All digester pH values remained stable and the effluent total VFA concentration averaged 48 ± 4 mg/L as acetic acid (n=8) (Appendix 4, Table 4A). The addition of PHB bioplastic as a co-digestate on Day 116 initially resulted in highly variable methane production in co-digesters (Figure 4.1). Subsequently, the methane production rate in all co-digesters increased by Day 160 as a result of PHB co-digestion.

Pretreating the PHB bioplastics at high pH and temperature reduced the lag time before PHB co-digestion commenced and increased methane production immediately after PHB began to be co-digested. The lag times were 3 to 5 d shorter for digesters fed pretreated versus untreated PHBs (Figure 4.1). The shorter lag times also resulted in higher cumulative methane production during the post-co-digestion transition period (days 116 to 135). Also, the cumulative methane production from pretreated PHBs was 4.4 to 6.8% higher than that from untreated PHBs during the transition period (Figure 4.1).

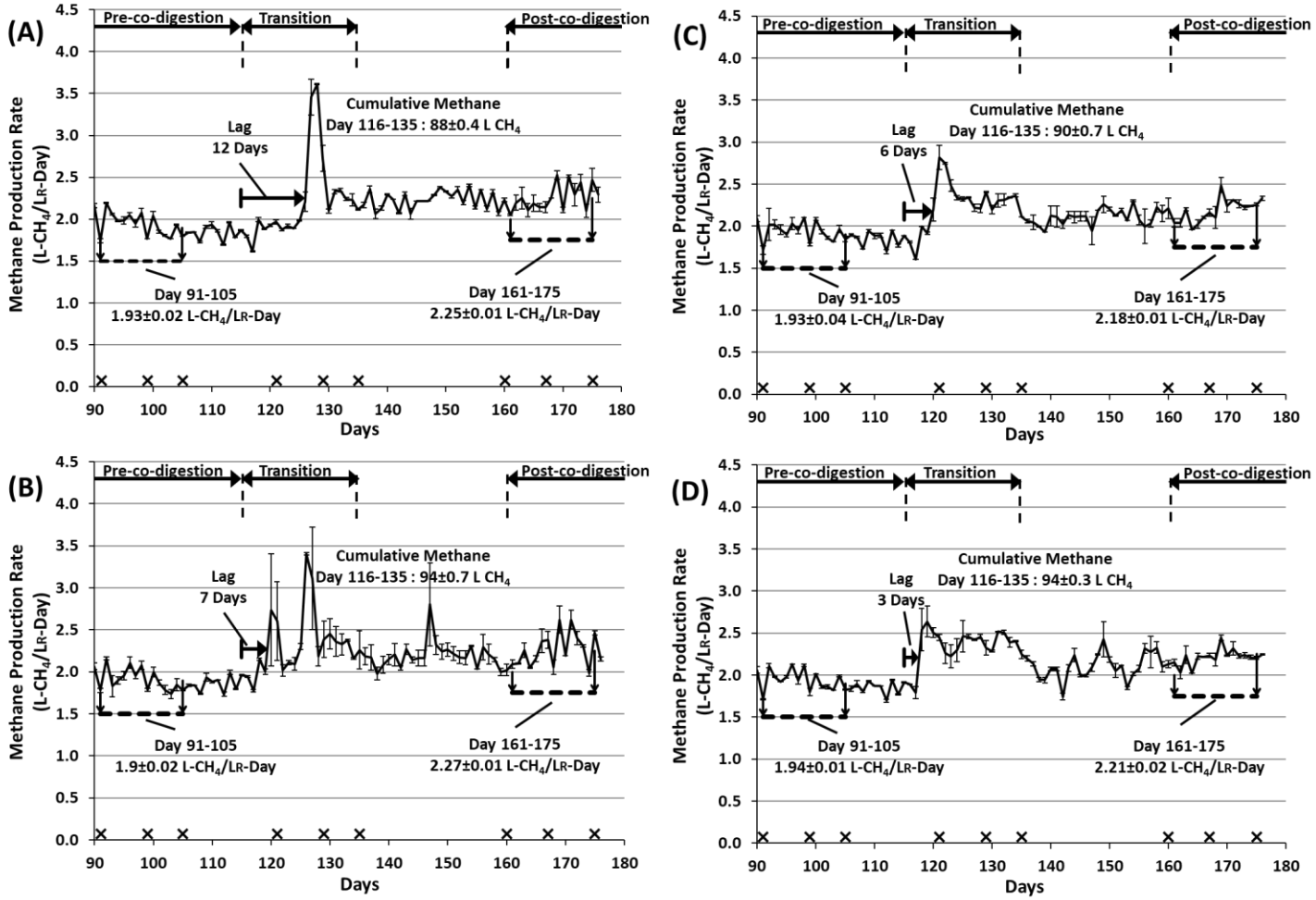

Figure 4.1 Digester average methane production at 35 °C co-digesting with synthetic municipal primary sludge (SMPS) and (A) untreated PHB1, (B) pretreated PHB1, (C) untreated PHB2, and (D) pretreated PHB2. Quasi steady-state periods before and after PHB co-digestion began are depicted at the top of each figure along with the transition period immediately after the start of PHB co-digestion. Sampling times for microbial community analysis are represented by "✕". Error bars are standard deviation (n=2).

Methane production during the post-co-digestion steady state period was 16% higher than that observed before bioplastics were co-fed. The total OLR when PHB was co-fed was 20% higher than when digesters were fed SMPS alone. Similar to the pre-co-digestion quasi steady state period, digester pH remained stable and the effluent VFA concentrations remained lower than 50 mg/L. In previous research, batch biochemical methane potential (BMP) testing over 40 d resulted in 50 to 80% and 82 to 100% PHB conversion to methane for raw and thermo-chemically pretreated PHB, respectively (Benn and Zitomer, 2018). Optimal thermochemical pretreatment of PHBs resulted in approximately 20% increases in BMP values; therefore, those pretreatments were used in this study. In 21-d batch experiments, Budwill et al. (1992) observed 87% conversion of PHB to methane and up to 96% conversion for a related PHA co-polymer. Similarly, Yagi et al. (2014) observed 92 to 93% conversion of PHB to methane during 26-d, batch anaerobic digestion. Therefore, the continuously-fed PHB co-digesters operating at 15-d HRT resulted in methane conversion efficiencies similar to those observed in previous batch experiments.

There was no long-term difference between methane production for untreated and pretreated PHB bioplastics during co-digestion. Methane production during the post-co-digestion quasi steady state period (days 160 to 175) for all digesters was similar (p value>0.05, n=8) and averaged 2.2 ± 0.02 L-CH_4/L-d. PHB conversion efficiency to methane during post-co-digestion quasi steady state was 93 ± 42 and 79 ± 21% for untreated PHB1 and PHB2, respectively, and 98 ± 4 and 84 ± 1% for pretreated PHB1 and PHB2, respectively. Duplicate digesters receiving pretreated PHB had notably less variation than those with untreated PHB during the quasi steady state. A 5 ± 0.1%

increase in PHB conversion efficiency was observed when PHBs were pretreated but this difference was not statistically significant. However, the most benefit from PHB pretreatment during co-digestion was related to a reduced lag time to attain quasi-steady state methane production, reducing this acclimation period by nearly 50%.

4.4.2 Microbial Community Analyses

Illumina sequencing yielded 15.5 million raw sequences, with 215,466 ± 55,825 (n=72) raw reads per sample. After 123,995 sequence reads (i.e., lowest sequence reads per sample), the number of OTUs was saturated as revealed by the asymptotic nature of the rarefaction curves and resulted in significant coverage. Therefore, a total of 8.7 million sequence reads from all 72 digesters samples were analyzed with 123,995 rarified sequence reads per sample. Based on 97% similarity, a total of 14,926 OTUs were observed with an average of 3503 ± 192 OTUs per sample.

The microbial community composition data from individual digesters during a given time period were more similar to each other than they were to microbial communities in other digesters as indicated by ANOSIM results (R=0.95, p=0.001). Alpha diversity indices such as richness, Shannon-Weaver diversity and evenness did not correlate with observed pre- or post-co-digestion digester methane production rates (Appendix 4, Figure 4A).

Digester microbial communities were significantly different before and after bioplastic co-digestion (Figure 4.2). Initially, all digester microbial communities were similar to each other during pre-co-digestion when SMPS without PHB was fed (ANOSIM R=0.55, p=0.001) (Figure 4.2). However, after PHB feeding commenced, the

microbial communities clustered separately from those of the pre-co-digestion period (ANOSIM R=0.82, p=0.001) (Figure 4.2).

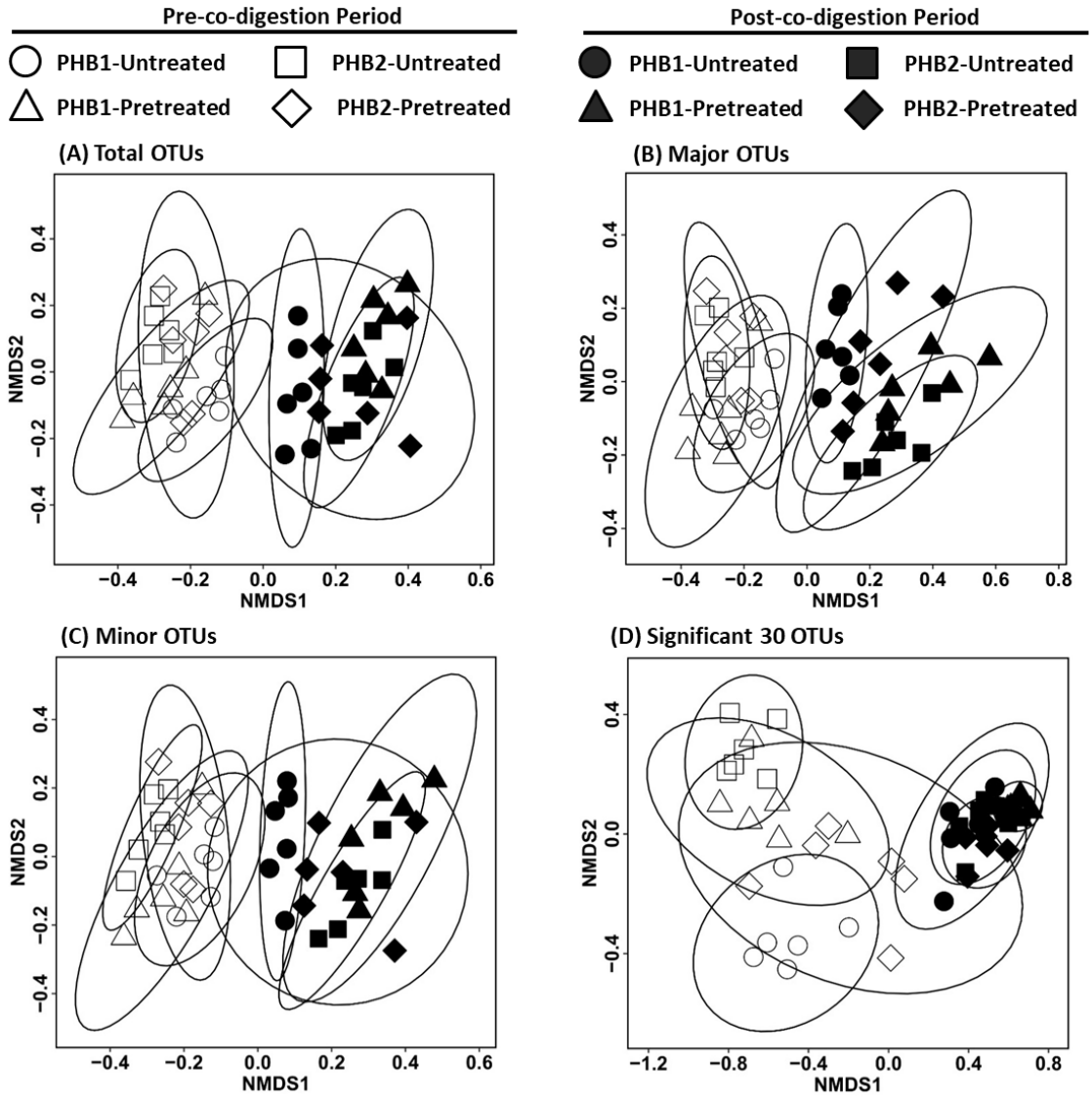

Figure 4.2 Digester microbial communities comparison NMDS plots during pre- and post-co-digestion periods based on (A) total microbial OTUs, (B) major OTUs (i.e., ≥0.1% relative abundance in at least one sample), (C) minor OTUs (i.e., <0.1% relative abundance) and (D) 30 significant OTUs having relative abundance values related to methane production rate using Spearman's rank correlation.

Factors such as the PHB type and whether or not the PHB was pretreated were not observed to affect the microbial community changes. Although the microbial

communities shifted after co-digestion started, all digester microbial communities converged during the post-co-digestion steady state period (ANOSIM R=0.61, p=0.001) (Figure 4.1).

A total of 366 major OTUs having ≥ 0.1% relative abundance in at least one sample were identified that accounted for 88.5 ± 0.7% of the total microbial abundance. The remaining 14,560 minor OTUs with lower (< 0.1%) relative abundance accounted for 11.5 ± 0.003% of the total abundance. Both major and minor OTU relative abundance values changed after PHB co-digestion began (Fig. 2B and C). The observed microbial community differences between pre- and post-co-digestion periods using major (ANOSIM R=0.83, p=0.001) and minor (ANOSIM R=0.81, p=0.002) OTU data were similar to that observed using total OTUs (Fig. 2A, B and C). Major shifts in microbial communities during co-digestion of municipal sewage solids and fat, oil and grease also have been reported due to change in the feed composition (Kurade et al., 2019).

4.4.2.1 Major Bacterial OTUs

Relative abundance values of major bacterial OTUs during pre- and post-co-digestion periods significantly changed after PHB bioplastic was fed to the co-digesters (Appendix 4, Figure 4B; ANOSIM R=0.87, p=0.001). The 342 major bacterial OTUs represented a total of 14 phyla. Relative abundance of two bacterial phyla significantly changed due to PHB co-digestion: the relative abundance of *Cloacimonetes* increased from 4.0 ± 1.8% to 8.8 ± 2.8% (p value<0.05, n=48) and *Chloroflexi* decreased from 2.8 ± 1.1% to 0.6 ± 0.2% (p value<0.05, n=48), respectively, from pre- to post-co-digestion periods. *Bacteroidetes* and *Firmicutes* were consistently the two most dominant phyla in

all co-digesters during both pre- and post-co-digestion periods, with major bacterial relative abundance values during the pre-co-digestion period of 35 ± 3.9% and 22 ± 2.0%, respectively; these values did not change significantly (p value>0.05, n=48) during post-co-digestion. Similarly, the relative abundance of phyla *Proteobacteria*, *Deferribacteres*, *Synergistetes*, *Thermotogae* and *Actinobacteria* did not change significantly (p value>0.05, n=48) from their pre-co-digestion values of 7.3 ± 1.1%, 5.8 ± 3.9%, 5.2 ± 1.1%, 3.0 ± 1.2% and 1.6 ± 0.6%, respectively.

4.4.2.2 Major Archaeal OTUs

There were 14 major archaeal OTUs observed in all samples. During the pre-co-digestion period, the combined relative abundance of the major archaeal OTUs ranged from 1.1 to 5.8%. The dominant archaeal OTU was most similar to *Methanosaeta* and accounted for 3.0 ± 1.2% of the total microbial abundance and 89.6 ± 3.4% of the total archaeal abundance during the pre-co-digestion period.

Despite the increase in OLR and methane production, PHB co-digestion had no significant influence on the archaeal community composition or archaeal relative abundance. No significant major archaeal OTU community change was observed after the digesters attained post-co-digestion quasi steady state period (Appendix 4, Figure 4B). The pre- and post-co-digestion archaeal community clustered together and were relatively similar (ANOSIM R=0.07, p=0.03). *Methanosaeta* remained the dominant archaeal OTU, accounting for 4.3 ± 2.2% of the total microbial community and 90.6 ± 6.7% of the total archaeal abundance during the post-co-digestion period.

Methanosaeta have a lower growth rate and higher affinity for acetate than the only other known acetoclastic methanogen genera (*Methanosarcina*). They typically outcompete *Methanosarcina* in digesters with low acetate concentration (< 500 mg/L) (Conklin et al., 2006; Hori et al., 2006). Since the co-digesters in this study had total VFA concentration of < 50 mg/L during pre- and post-co-digestion periods, the presence of *Methanosaeta* as the dominant acetoclastic methanogen was reasonable.

4.4.2.3 Spearman Correlation to Select Significant OTUs

Major OTUs of 48 digester samples (24 from pre- and post-co-digestion period, respectively) were correlated with the observed methane production rate on the days the samples were taken. Spearman's rank order correlation analysis yielded 30 significant OTUs with relative abundance values correlating to co-digester methane production (Figure 4.2D). All significant OTUs were bacteria, whereas no archaea were identified. Of the 30 significant OTUs, 16 were positively correlated and 14 were negatively correlated with methane production (Table 43). Though the archaeal community is important for a stable functioning digester, the results indicates that the bacterial community may have played a more crucial role, as bacterial hydrolysis is ostensibly the rate limiting step during PHB co-digestion.

Table 4.1 Blast search result of the Spearman correlated 30 significant OTUs. Of the 30 selected OTUs, 16 OTUs were positively and 14 OTUs were negatively correlated with methane production. Taxonomic classification in bold font represent the valid level based on percent homology with the homology percentage ranges in parentheses. Relative abundance ranges and averages are for 24 samples.

	OTU #	Phylum (> 77 %)	Class (80 - 85 %)	Order (85 - 90 %)	Family (90 - 95 %)	Genus (> 95 % Homology)	Percent Homology	Pre-Co-digestion Relative Abundance Range & (Avg), %	Co-digestion Relative Abundance Range & (Avg), %
Positively Correlated OTUs	OTU 1	Deferribacteres	Deferribacteres	Deferribacterales	**Deferribacteraceae**	*Deferribacter*	91.4	0.01 to 0.08 (0.05)	0.06 to 0.15 (0.10)
	OTU 2	Deferribacteres	Deferribacteres	Deferribacterales	**Deferribacteraceae**	*Deferribacter*	91.9	0.02 to 0.07 (0.04)	0.04 to 0.13 (0.09)
	OTU 3	Deferribacteres	Deferribacteres	Deferribacterales	**Deferribacteraceae**	*Deferribacter*	92.3	0.29 to 1.27 (0.84)	1.01 to 2.41 (1.75)
	OTU 4	Deferribacteres	Deferribacteres	Deferribacterales	**Deferribacteraceae**	*Deferribacter*	92.1	0.02 to 0.07 (0.05)	0.06 to 0.14 (0.10)
	OTU 5	Deferribacteres	Deferribacteres	Deferribacterales	**Deferribacteraceae**	*Deferribacter*	90.4	0.12 to 0.49 (0.33)	0.39 to 0.96 (0.69)
	OTU 6	Deferribacteres	Deferribacteres	Deferribacterales	**Deferribacteraceae**	*Deferribacter*	92.4	0.02 to 0.09 (0.05)	0.06 to 0.16 (0.11)
	OTU 7	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	***Geobacter*** [1]	98.5	0.01 to 0.04 (0.02)	0.03 to 0.12 (0.07)
	OTU 8	Firmicutes	Clostridia	Clostridiales	**Gracilibacteraceae**	*Gracilibacter*	92.2	<0.01 to 0.0 (0.0)	<0.01 to 0.17 (0.04)
	OTU 9	Firmicutes	Clostridia	Clostridiales	**Gracilibacteraceae**	*Gracilibacter*	91.2	0.03 to 0.07 (0.04)	0.06 to 1.69 (0.28)
	OTU 10	Firmicutes	Clostridia	Clostridiales	**Gracilibacteraceae**	*Gracilibacter*	93.0	<0.01 to 0.01 (0.01)	0.02 to 0.39 (0.10)
	OTU 11	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	***Kosmotoga*** [2]	99.6	0.49 to 2.28 (1.23)	2.16 to 5.06 (3.26)
	OTU 12	Firmicutes	**Negativicutes**	Selenomonadales	Veillonellaceae	*Pelosinus*	84.3	<0.01 to 0.01 (0.0)	0.06 to 0.21 (0.12)
	OTU 13	Firmicutes	Clostridia	Clostridiales	Clostridiales	***Pseudoflavonifractor***	96.0	<0.01 to 0.01 (0.0)	<0.01 to 0.16 (0.04)
	OTU 14	Firmicutes	Clostridia	Clostridiales	Clostridiales	***Pseudoflavonifractor*** [3]	97.1	<0.01 to 0.0 (0.0)	<0.01 to 0.03 (0.01)
	OTU 15	Firmicutes	Clostridia	Clostridiales	**Ruminococcaceae**	*Ruminococcus*	93.4	0.01 to 0.07 (0.03)	0.05 to 0.42 (0.12)
	OTU 16	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	**Syntrophorhabdaceae**	*Syntrophorhabdus*	94.5	0.04 to 0.08 (0.06)	0.05 to 0.2 (0.11)
Negatively Correlated OTUs	OTU 17	Bacteroidetes	**Bacteroidia**	Bacteroidales	Bacteroidaceae	*Bacteroides*	84.7	0.02 to 1.35 (0.55)	<0.01 to 0.04 (0.01)
	OTU 18	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	***Bellilinea***	96.3	0.21 to 1.75 (0.78)	0.03 to 0.27 (0.13)
	OTU 19	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	***Bellilinea***	96.1	0.01 to 0.11 (0.04)	<0.01 to 0.02 (0.01)
	OTU 20	Firmicutes	Clostridia	Clostridiales	**Clostridiaceae**	*Clostridium*	93.0	0.01 to 0.10 (0.04)	<0.01 to 0.02 (0.01)
	OTU 21	Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	***Deferribacter*** [4]	99.6	0.22 to 0.61 (0.39)	0.07 to 0.22 (0.15)
	OTU 22	Firmicutes	Clostridia	**Clostridiales**	Eubacteriaceae	*Eubacterium*	86.8	0.11 to 2.23 (0.68)	0.03 to 0.25 (0.11)
	OTU 23	Proteobacteria	Gammaproteobacteria	Chromatiales	Halothiobacillaceae	*Halothiobacillus*	73.6	<0.01 to 0.16 (0.04)	<0.01 to 0.02 (0.0)
	OTU 24	Planctomycetes	Planctomycetia	**Planctomycetales**	Planctomycetaceae	*Planctomyces*	87.5	0.02 to 0.53 (0.17)	<0.01 to 0.08 (0.03)
	OTU 25	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	***Proteiniphilum*** [5]	98.5	0.02 to 0.10 (0.05)	<0.01 to 0.03 (0.02)
	OTU 26	Proteobacteria	Gammaproteobacteria	**Alteromonadales**	Pseudoalteromonadaceae	*Pseudoalteromonas*	89.1	0.24 to 6.97 (3.05)	0.07 to 1.23 (0.24)
	OTU 27	Proteobacteria	Gammaproteobacteria	**Alteromonadales**	Pseudoalteromonadaceae	*Pseudoalteromonas*	85.7	<0.01 to 0.16 (0.06)	<0.01 to 0.02 (0.0)
	OTU 28	Bacteroidetes	**Sphingobacteriia**	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	84.2	0.04 to 1.63 (0.69)	<0.01 to 0.05 (0.02)
	OTU 29	Firmicutes	Clostridia	Clostridiales	Symbiobacteriaceae	***Symbiobacterium***	95.6	0.03 to 0.20 (0.06)	<0.01 to 0.06 (0.02)
	OTU 30	Firmicutes	**Clostridia**	Thermoanaerobacterales	Thermoanaerobacterales family iii. incertae sedis	*Thermovenabulum*	83.5	0.15 to 0.99 (0.44)	0.01 to 0.41 (0.08)

[1] > 97 % homology; *uncultured Geobacter sp.*

[2] > 97 % homology; ay692052.1 UASB reactor clone m79

[3] > 97 % homology; *uncultured Pseudoflavonifractor sp.*

[4] > 97 % homology; *uncultured deferribacter sp.*

[5] > 97 % homology; *uncultured proteiniphilum sp.*

Previous studies on anaerobic digestion of complex carbon substrates have also resulted in similar findings. Yue et al. (2013) reported a significant shift in digester bacterial community, compared to the archaeal community, when the substrate composition changed after co-digestion of cattle manure with corn stover was initiated. Conversely, both bacterial and archaeal communities changed significantly when only the SRT value of the co-digesters was varied (Yue et al., 2013). Similarly, Ziganshin et al. (2013) reported that bacterial communities were influenced significantly by varying substrate composition during anaerobic co-digestion of cattle manure with various agricultural residues (chicken manure, distillers grain, maize silage, maize straw and jatropha cake), and both bacterial and archaeal communities were influenced by other factors, such as digester operating temperature, SRT and organic loading rate.

OTUs most similar to *Kosmotoga* and *Deferribacter* became more dominant after PHB co-digestion, as indicated by relative abundance values (Table 43). The taxonomic identification of the positively correlated bacterial OTUs were distinct from the negatively correlated OTUs. Except for one negatively correlated OTU of genus *Deferribacter* (OTU 21), the genera of the 13 remaining negatively correlated OTUs were not represented among the 16 positively correlated OTUs (Table 43).

The significant OTU relative abundance values were less similar (ANOSIM $R=0.91$, $p=0.001$) than those of the major bacterial OTUs when comparing pre- and post-co-digestion quasi steady state periods (Figure 4.2 B, D). Relative abundance heatmap with dual hierarchical clustering of the 30 significant OTUs illustrates a major shift from pre to post-co-digestion (Appendix 4, Figure 4C). Taxa with relative abundance values that positively (OTUs 1–16) and negatively (OTUs 17–30) correlated with methane

production clustered into two branches. Likewise, pre and post-co-digestion samples clustered into two distinct branches with post-co-digestion samples primarily clustered by presence or absence of pre-treatment, but not by PHB type. Pre-co-digestion samples showed no clustering pattern. Sample clustering depicted a clear differentiation between pre and post-co-digestion communities and the influence of PHB treatment on microbial community composition. The combined relative abundance of the 16 positively correlated OTUs increased from 2.8 ± 0.6% during pre-co-digestion to 7.1 ± 1.2% during post-co-digestion. Conversely, the combined relative abundance of the 14 negatively correlated OTUs decreased from 7.2 ± 3.0% during pre-co-digestion to 0.8 ± 0.3% during the co-digestion period.

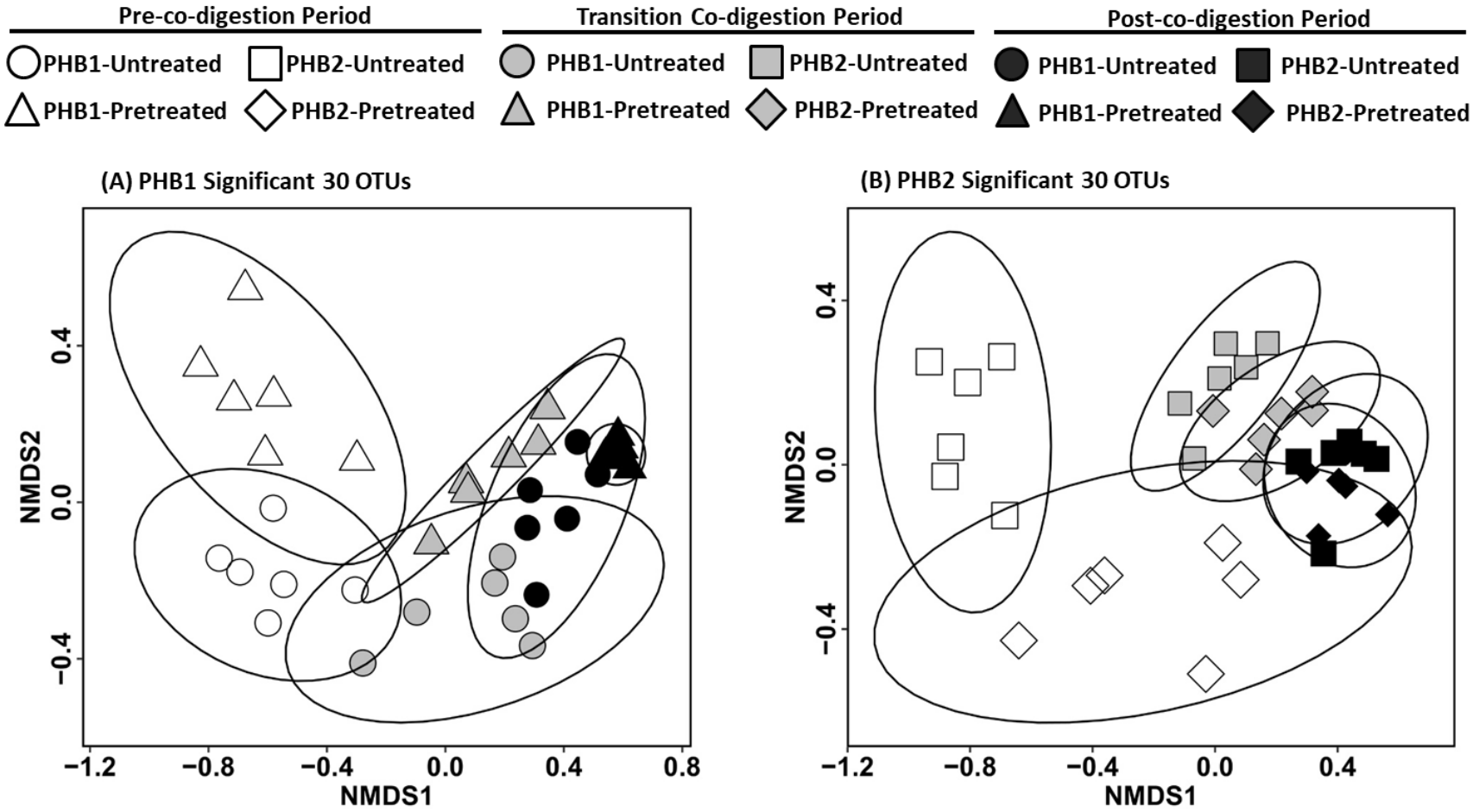

Figure 4.3 Digester microbial communities comparison NMDS plots during pre-, transition-, and post-co-digestion periods for digesters receiving (A) PHB1 and (B) PHB2 based on the 30 significant OTUs having relative abundance values related to methane production rate using Spearman's rank correlation.

Relative abundance values of significant OTUs changed and converged for all digesters after each bioplastic was co-digested (Figure 4.3 A, B). Pre- and post-co-digestion quasi steady state and transition period relative abundance value similarity was quantified using the ANOSIM statistic value (R) and employed the significant OTU data (Appendix 4, Table 4B). Co-digesters were able to more quickly adapt and exhibited shorter lag times when pre-co-digestion communities were less similar to transition period communities (Figure 4.4A) and when transition communities were more similar to post-co-digestion communities (Figure 4.4C). In addition, cumulative transition period methane production was higher when pre-co-digestion and transition period communities were less similar (Figure 4.4B), or when transition versus post-co-digestion communities were more similar (Figure 4.4D). Conversely, co-digesters with shorter lag times and higher cumulative transition period methane production showed more similarity between post-co-digestion and transition period communities. The community shift in the 342 major bacterial OTUs during pre-, transition- and post-co-digestion periods were also compared with the observed difference in lag time and cumulative transition period methane production. However, the shift in the major bacterial OTUs did not show a strong correlation like that observed using the 30 significant OTUs (Appendix 4, Table 4B).

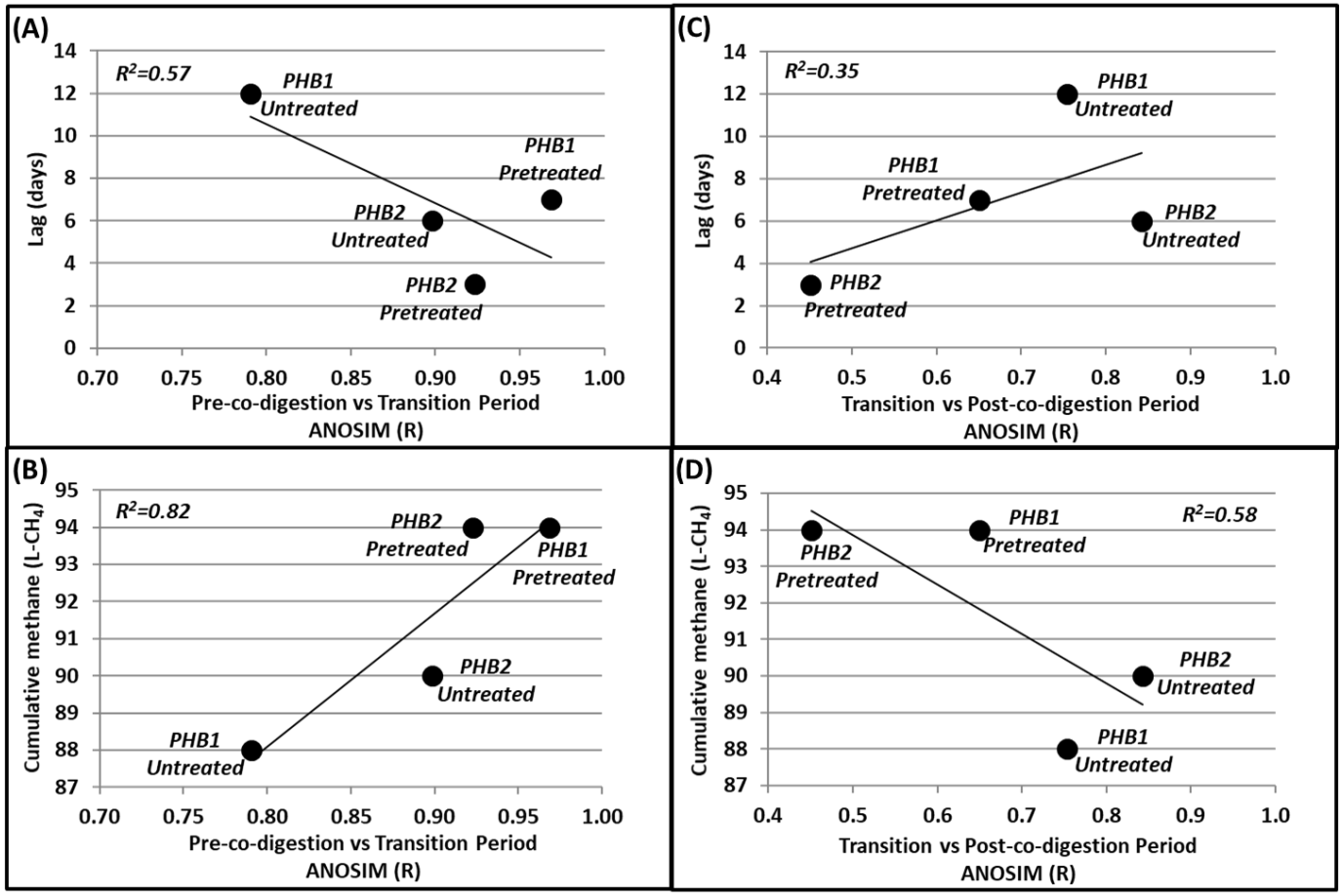

Figure 4.4 Similarity of pre-co-digestion and transition period microbial communities versus (A) lag time before post-co-digestion PHB methane production commenced and (B) cumulative methane produced during the transition period. Similarity between transition and post-co-digestion period microbial communities versus (A) lag time before post-co-digestion PHB methane production commenced and (B) cumulative transition period methane produced. Community similarity was quantified by the ANOSIM statistic value (R). ANOSIM was performed using the 30 significant OTUs having relative abundance values that related to methane production rate using Spearman's rank correlation.

Factors such as the change in OLR brought about by PHB co-digestion could influence the microbial community due to potential changes in the digester pH, VFA concentration or other parameters. However, the OLR increased only 20% during PHB co-digestion, and no drop in pH or high VFA production (< 50 mg/L) was observed after bioplastic co-digestion. Substrate composition is also known to influence microbial community composition in anaerobic digesters (Álvarez et al., 2010; Cesaro and Belgiorno, 2014; Noike et al., 1985). Therefore, it is more likely that change in significant OTU relative abundance was due to the change in substrate composition after co-digestion started rather than due to OLR increase.

4.4.3 The Role of Positively Correlated OTUs in Anaerobic PHB Degradation

Of the known PHA or PHB degrading bacteria, only the genera *Streptomyces* and *Bacillus* were most similar to OTUs identified in this study (Abou-Zeid et al., 2001; Budwill et al., 1996; Emadian et al., 2017; Janssen and Harfoot, 1990; Mergaert et al., 1996). However, the relative abundance values of *Streptomyces* and *Bacillus* OTUs were relatively low (< 0.001%) and did not significantly increase after PHB addition. In addition, none of the 16 positively correlated OTUs were previously reported to have a role in PHB degradation. Microbial degradation of bio-polymers such as PHB or PHA requires extracellular enzymes such PHA depolymerase and lipase (Banerjee et al., 2014; Rodríguez-Contreras et al., 2012). Taxa to which the 30 significant OTUs were most similar were compared to a current list of microorganisms that possess PHA depolymerase or lipase enzymes (Knoll et al., 2009; Pleiss et al., 2000). None of the 30 significant OTUs were found in the PHA depolymerase database. Two positively

correlated OTUs, *Geobacter* and *Ruminococcus* were found in the lipase database; however, so were six of the negatively correlated OTUs: *Bacteroides*, *Clostridium*, *Eubacterium*, *Planctomyces*, *Pseudoalteromonas,* and *Symbiobacterium*.

Several of the positively correlated significant OTUs have been previously identified for their fermentative and acetogenic function during anaerobic digestion. *Deferribacter* and *Pseudoflavonifractor* are known acidogenic amino acid degraders (Cardinali-Rezende et al., 2016; Jumas-Bilak et al., 2009; Talbot et al., 2008). *Geobacter* and *Syntrophorhabdus* are known for direct interspecies electron transfer (DIET) in anaerobic digestion and generally are important syntrophic bacteria co-occurring symbiotically with hydrogenotrophic methanogens (McInerney et al., 2007; Shen et al., 2016). *Gracilibacter thermotolerans*, the only *Gracilibacter* taxa characterized, is defined as acidogenic, obligate anaerobe and ferments a number of carbohydrates yielding acetate, lactate and ethanol (Lee et al., 2006). Members of the order *Thermotogales*, including *Kosmotoga*, *Fervidobacterium* and *Geotoga*, are well characterized carbohydrate hydrolyzers and fermenters, and proliferate in anaerobic digesters (Ju et al., 2017; Peces et al., 2018; P. Wang et al., 2018). *Pelosinus* is a strict anaerobe and has been associated with acetogenic fermentation of lactate through the expression of hydrolyzing lipase enzymes (Jaeger et al., 1995; Roohi et al., 2018). Members of the genera *Ruminococcus* are anaerobic and cellulolytic bacteria which play an important role in the hydrolysis and fermentation of hemi-cellulosic and cellulosic materials during anaerobic digestion (Yi et al., 2014).

Most current knowledge regarding PHB-degrading microorganisms is based on isolates from natural environments such as soil or river sediments contaminated by PHB.

In contrast, reports regarding microbial communities during anaerobic co-digestion of PHB are lacking. The results of this study show that anaerobic co-digestion of PHB and SMPS significantly influences the relative abundance of specific bacteria that are have not been previously identified to be involved in PHB degradation. In addition, none of the currently known PHA degrading microorganisms was observed to play a significant role in anaerobic PHB co-digestion. Therefore, there may be as-yet-unknown PHB degrading bacteria. The hydrolytic and lipolytic activities of the diverse bacterial community in an anaerobic digester treating primary sludge are sufficient to co-digest PHB polymers. The results of this study confirm that municipal water reclamation facilities with excess capacity could co-digest PHB bioplastic in addition to municipal wastewater sludge to generate more methane and renewable energy. Furthermore, pretreatment of bioplastics at high temperature and pH can further help by decreasing lag time and increasing methane production immediately after PHB bioplastic co-digestion is initiated.

New insights into the microbial community of PHB co-digesters can advance sustainable bioplastic waste management strategies. Fundamental knowledge of the complex microbial consortia needed for successful PHB co-digestion is useful for monitoring startup operations when full-scale bioplastic co-digestion is initiated. Troubleshooting full-scale bioplastic co-digestion can also be accomplished through observation of the microbial community and may help to predict lag time associated with community acclimation. Predicting methane production rate from relative abundance data of the significant OTUs identified herein may further improve co-digester design and in the selection of co-digester inoculum in the future (Venkiteshwaran et al., 2017).

4.5 Conclusions

Methane production increased resultant of PHB co-digestion with no change in digester performance. Pretreatment of PHB bioplastic at high pH and temperature initially reduced the lag time before methane production increased when PHB co-digestion began. PHB co-digester bacterial communities changed, whereas no archaeal community change was observed.

No previously known PHB degraders were observed in the co-digesters. OTUs most similar to *Deferribacter*, *Geobacter*, *Kosmotoga*, and *Ruminococcus* were found to correlate positively with increased methane production resulting from PHB co-digestion. These OTUs may play an important role in PHB bioplastic conversion to methane in anaerobic digestion.

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5 Overall Conclusions and Recommendations

The motivation for this study emerged from environmental problems caused by unsustainable production and accumulation of conventional non-biodegradable plastic. One prospective route to a more sustainable plastic economy involves a cradle-to-cradle scenario in which a bioplastic, biologically produced from methane, is anaerobically digested to biomethane for either renewable energy or closed-loop recycling.

The overarching goal of this work was to investigate and develop a system for biomethane production from biodegradable plastic on a time scale compatible with anaerobic co-digestion. To accomplish this, first bioplastics were selected, processing and pretreatment methods were standardized, and biochemical methane potentials were determined according to hypothesis 1 objectives. BMP experiments evaluating bioplastic pretreatment conditions showed PHB, rather than PLA, was more amenable to mesophilic anaerobic digestion. Untreated PHBs yielded between 50 – 80% of theoretical methane potential in 40 d, whereas untreated PLA did not produce biomethane even when the test was extended to 60 d. However, PLA yielded up to 22% of theoretical methane potential when pretreated at 90 °C regardless of pH and proportional to treatment time, and the lag time reduced to less than one day. Indeed, PLA pretreatment could be further optimized for digestion or other recycle techniques but was outside the scope of this study. Further, PHB pretreatment conditions generally 35 or 55 °C, pH greater than neutral and 24 or 48 h of treatment time yielded between 82 – 100% of theoretical methane potential in 40 d, representing 2 – 100% increase compared to untreated. Statistically significant increases in BMP from optimal pretreatment were observed for all bioplastics tested except for methane-derived PHB3. However, PHB3

was the most readily biodegradable untreated bioplastic in terms of methane yield, suggesting no requirement for pretreatment. Lag times for optimal pretreatment conditions of PHBs ranged from 4 – 16 d. PHB1 and PHB2 showed the largest increase in BMP when optimally pretreated and were thus chosen for continuous feed bench-scale co-digestion.

Co-digesters treating synthetic primary sludge were seeded from a municipal digester and both untreated and pretreated (55°C, pH 12) PHB1 and PHB2 were co-fed at 20% of OLR operating at 15 d HRT/SRT according to hypothesis 2 objectives. Pretreatment of PHBs helped to reduce lag time by approximately 40% from 9 to 5 d compared to untreated PHBs, though statistical significance could not be evaluated due to limited replicates. At steady state, co-digestion biomethane yield represented 80 – 98% of theoretical methane production from PHB and pretreatment increased conversion efficiency by 5% compared to untreated PHBs. Post- PHB co-digester effluent VFA, pH, % biomethane in biogas, and VS removal remained stable at steady state. All digesters were statistically different pre- vs post-co-digestion, so PHB as a co-substrate significantly increased methane production whether untreated or pretreated and regardless of PHB type. Both untreated PHB post-co-digestion duplicate digesters were statistically different from one another, whereas both pretreated PHB post-co-digestion duplicate digesters were statistically similar in terms of methane production. This suggests pretreatment of PHB increases reproducibility of PHB co-digestion at quasi-steady state after 3 SRTs. However, despite a 5% increase in duplicate average PHB conversion efficiency to methane for both untreated vs pretreated PHBs, there was no statistically significant methane production difference between duplicate untreated and

pretreated PHB co-digesters for both PHBs tested. This statistically indistinguishable difference originated from poor reproducibility among untreated PHB post-co-digesters. Therefore, more replicate PHB co-digesters or longer test durations are necessary for future experiments. Overall, PHB to biomethane conversion efficiency was similar to other research findings and proportionate with previous BMP experiments.

Further, the microbial community compositions were compared using Illumina DNA sequencing of the 16S rRNA gene during steady-state pre- and post-co-digestion of PHBs to help unravel intricate microbial associations within anaerobic digesters performing PHB biodegradation. Archaeal communities were not observed to change significantly comparing pre- and post-co-digestion of PHB. On the other hand, bacterial communities exhibited major changes when PHB was co-digested. Bacterial OTUs demonstrating the greatest degree of rank order correlation (Spearman) to increased methane production due to PHB were most similar to *Deferribacter*, *Geobacter*, *Kosmotoga*, and *Ruminococcus*. However, previously known PHB degraders were not observed in this correlation. Thus, these OTUs may harbor or enable specific functionality during methanogenic co-digestion of PHB.

In summary, PHB bioplastic pretreatment is not necessary for successful co-digestion but ostensibly can offer reduced lag time, at least 5% increased conversion to methane, and improved reproducibility. Conversely, PLA bioplastic required extensive pretreatment for only partial conversion to methane and is not compatible with mesophilic anaerobic digestion. Both types of bioplastics investigated required size reduction processing to achieve reproducible results. Microbial communities of PHB co-digestion revealed valuable OTU correlations but causative microbial relationships were

not established. Nonetheless, the feasibility of anaerobically digesting bioplastic in a relevant waste management scenario was demonstrated.

Although considerable evidence supporting PHB co-digestion was presented here, there remain areas for continued research and development. Collection and separation of bioplastics could be a major challenge, requiring public outreach and education, new techniques or technologies for separation, etc. However, one promising scenario involving food waste mixed with bioplastic packaging and coupled with compost collection systems could help to lessen collection and separation hurdles by utilizing preexisting systems and eliminating separation. Yet little work has been done for co-digestion of bioplastics and food waste or organic fraction of municipal waste and practical challenges remain. Size reduction of bioplastics prior to co-digestion is ostensibly vital for rapid conversion to biomethane, but methods used in this study have not been scaled-up for feasible implementation. Therefore, grinding bioplastics to adequately sized particles, whether wet or dry requires further development.

In addition, the more widely available PLA bioplastics tested in this study were not compatible with common completely mixed mesophilic anaerobic digestion, but thermal pretreatment gave promising results and should be investigated further. Lastly, it is important to recognize that bioplastic feedstock, production methods, and end-of-life options all contribute massively to their sustainability profile, so implementing systems that prioritize and incentivize sustainable management throughout product lifecycles is important.

Appendices

Appendix 3

The appendix to "3 PRETREATMENT and ANAEROBIC CO-DIGESTION of SELECTED PHB and PLA BIOPLASTICS" contains the following tables and figures: Bench scale co-digestion pH **(Figure 3A)**, Bench scale co-digestion VFAs **(Figure 3B)**, Basal nutrient media **(Table 3A)**, PHB1 BMP results **(Table 3B)**, PHB2 BMP results **(Table 3C),** PHB3 BMP results **(Table 3D),** PHB4 BMP results **(Table 3E)**, and PLA BMP results **(Table 3F)**.

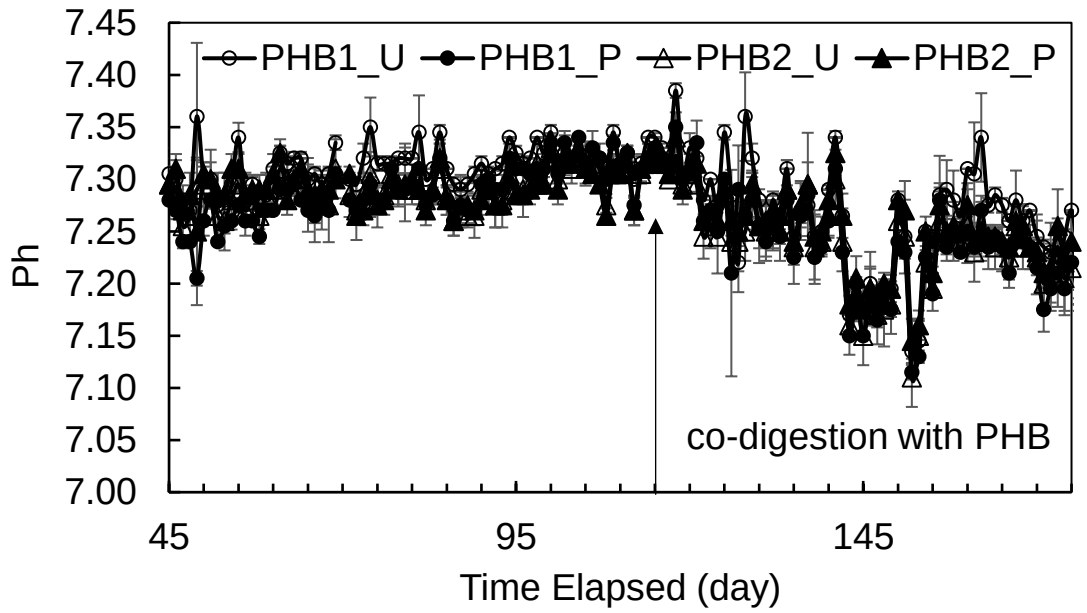

Figure 3A Bench scale co-digestion pH for duplicate digesters receiving PHB1 and PHB2 with "U" for untreated and "P" for pretreated; arrow indicates day 115 when PHB co-digestion began. Error bars show standard deviation of duplicate digesters.

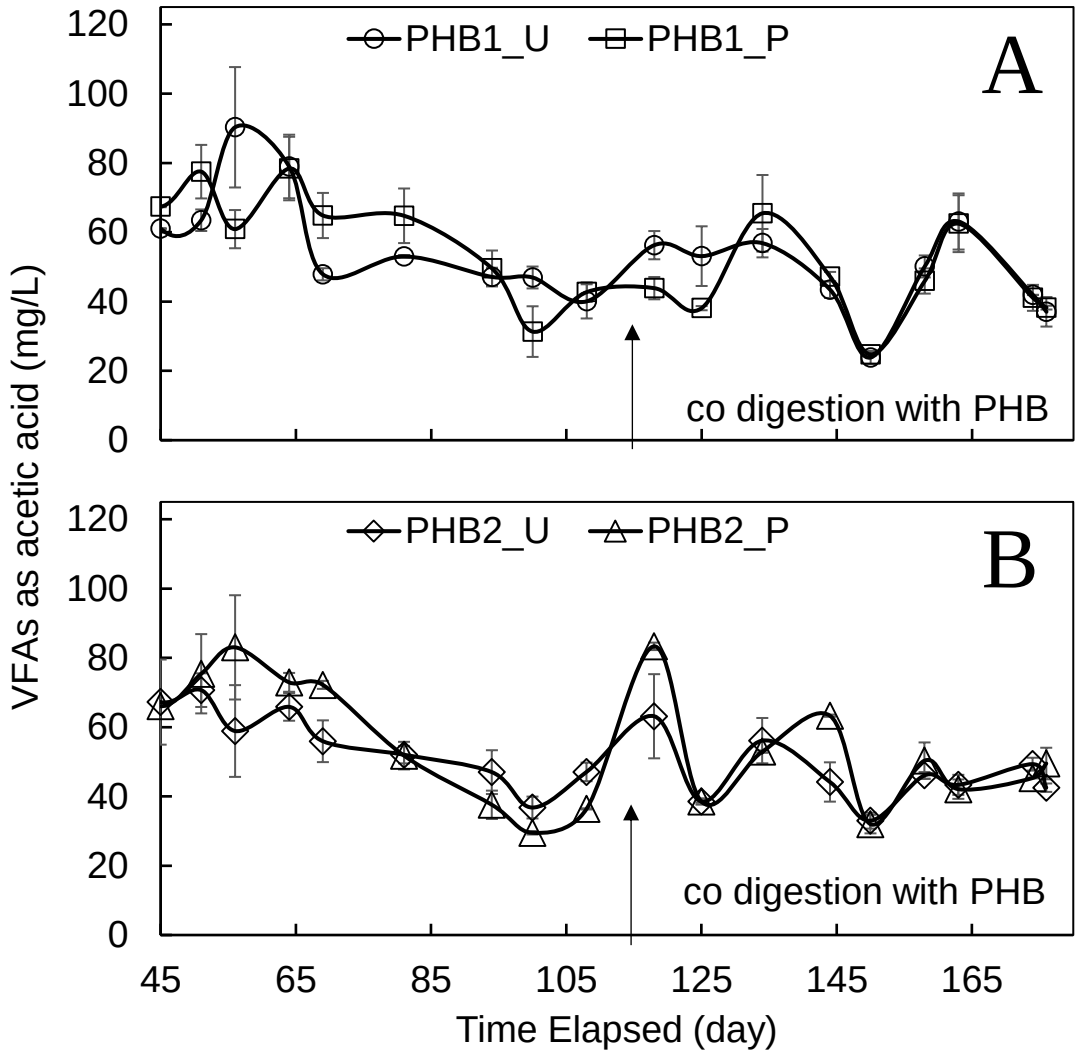

Figure 3B Bench scale co-digestion VFAs as acetic acid equivalents for digesters receiving PHB1 (A) and PHB2 (B) with "U" for untreated and "P" for pretreated; arrow indicates day 115 when PHB co-digestion began. Error bars show standard deviation of duplicate digesters.

Table 3A Basal nutrient media modified from original reported by (Speece, 2008).

Constituent	**Concentration in Reactor** (mg/L)
NH_4Cl	400
$MgSO_4 * 7H_2O$	195
KCl	400
$CaCl_2 * 2H_2O$	50
$(NH_4)_2HPO_4$	80
$FeCl_2 * 4H_2O$	10
$CoCl_2 * 6H_2O$	1
KI	10
$(NaPO_3)_6$	10
$NiCl_2 * 6H_2O$	1
$ZnCl_2$	1
$MnCl_2 * 4H_2O$	0.5
NH_4VO_3	0.5
$CuCl_2 * 2H_2O$	0.5
$AlCl_3 * 6H_20$	0.5
$NaMoO_4 * 2H_2O$	0.5
H_3BO_3	0.5
$NaWO_4 * 2H_2O$	0.5
Na_2SeO_3	0.5
$NaHCO_3$	6000
$Na_2S * 9H_2O$	300
L-Cysteine	10
*Yeast Extract	10

* not included in original

Table 3B PHB1 BMP results (mL CH_4/g ThOD), percentage of untreated control (NC), and Mann-Whitney test p value table for PHB1 thermal pretreatment (**A**, **B**) and thermal alkaline pretreatment (**C**); thermal pretreatment BMP ID denotes "pretreatment temperature (°C) pretreatment duration (h)"; thermal alkaline pretreatment BMP ID denotes "pretreatment temperature (°C)_pH_pretreatment duration (h)"; glucose positive control (PC).

Statistically Significant Difference < 0.05

Maximum BMP

A

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
90_3	208	9	-31	0.985
90_24	307	20	2	0.500
90_48	350	17	16	0.040
35_3	283	19	-6	0.905
35_24	348	27	15	0.040
35_48	**407**	**49**	**35**	**0.040**
NC	301	17		0.590

B

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
55_3	308	4	-13	0.866
55_24	364	9	3	0.669
55_48	**319**	**25**	**-10**	**0.905**
NC	353	43		0.590
PC	262	9		

C				
BMP ID	40 Day BMP	Stdev	% diff NC	p-value
35_8_3	337	10	69	0.095
35_8_24	348	17	74	0.040
35_8_48	349	2	75	0.040
35_10_3	377	65	89	0.040
35_10_24	341	50	71	0.095
35_10_48	355	18	78	0.040
35_12_3	382	4	92	0.040
35_12_24	**398**	**27**	**100**	**0.040**
35_12_48	365	41	83	0.040
55_8_3	377	33	89	0.040
55_8_24	359	5	80	0.038
55_8_48	333	61	67	0.095
55_10_3	267	43	34	0.669
55_10_24	319	28	60	0.191
55_10_48	337	24	69	0.095
55_12_3	365	8	83	0.040
55_12_24	393	33	97	0.040
55_12_48	360	11	81	0.040
NC	199	161		0.590
PC	237	26		

Table 3C PHB2 BMP results (mL CH_4/g ThOD), percentage of untreated control (NC), and Mann-Whitney test p value table for PHB2 thermal pretreatment (**A**) and thermal alkaline pretreatment (**B**); thermal pretreatment BMP ID denotes "pretreatment temperature (°C) pretreatment duration (h)"; thermal alkaline pretreatment BMP ID denotes "pretreatment temperature (°C)_pH_pretreatment duration (h)"; glucose positive control (PC).

Statistically Significant Difference < 0.05
Maximum BMP

A

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
35_3	280	45	20	0.191
35_24	311	47	34	0.095
35_48	**359**	**25**	**54**	**0.040**
55_3	311	30	34	0.095
55_24	340	11	46	0.040
55_48	287	45	23	0.191
90_3	314	36	35	0.095
90_24	301	32	29	0.095
90_48	292	44	25	0.191
NC	233	57		0.590

B

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
55_8_3	71	9	7	0.191
55_8_24	58	2	-12	0.977
55_8_48	64	6	-4	0.669
55_10_3	71	11	6	0.500
55_10_24	66	4	-1	0.331
55_10_48	58	4	-13	0.985
55_12_3	65	5	-2	0.588
55_12_24	124	36	86	0.040
55_12_48	**133**	**6**	**99**	**0.040**
90_8_3	69	11	3	0.412
90_8_24	53	6	-20	0.985
90_8_48	50	6	-25	0.987
90_10_3	52	7	-23	0.985
90_10_24	58	4	-14	0.985
90_10_48	59	2	-12	0.977
90_12_3	103	3	55	0.040
90_12_24	94	2	41	0.040
90_12_48	104	8	55	0.040
NC	67	9		0.590

Table 3D PHB3 BMP results (mL CH_4/g ThOD), percentage of untreated control (NC), and Mann-Whitney test p value table for PHB3 thermal pretreatment (**A**) and thermal alkaline pretreatment (**B**); thermal pretreatment BMP ID denotes "pretreatment temperature (°C) pretreatment duration (h)"; thermal alkaline pretreatment BMP ID denotes "pretreatment temperature (°C)_pH_pretreatment duration (h)"; glucose positive control (PC).

Statistically Significant Difference < 0.05
Maximum BMP

A

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
35_3	**159**	**166**	**9**	**0.412**
35_24	63	136	-57	0.809
35_48	63	150	-57	0.669
55_3	129	138	-12	0.866
55_24	147	156	1	0.669
55_48	142	152	-3	0.669
90_3	126	138	-14	0.905
90_24	126	122	-14	0.669
90_48	106	174	-28	0.500
NC	146	153		0.590
PC	438	71		

B

BMP ID	50 Day BMP	Stdev	% diff NC	p-value
55_8_3	329	10	0	0.614
55_8_24	336	3	2	0.331
55_8_48	334	13	1	0.331
55_10_3	333	13	1	0.412
55_10_24	**343**	**8**	**4**	**0.095**
55_10_48	320	14	-3	0.809
55_12_3	329	11	0	0.500
55_12_24	336	8	2	0.331
55_12_48	299	#DIV/0!	-9	0.963
90_8_3	332	4	1	0.500
90_8_24	323	9	-2	0.669
90_8_48	339	7	3	0.253
90_10_3	342	3	4	0.095
90_10_24	317	15	-4	0.905
90_10_48	274	39	-17	0.960
90_12_3	291	27	-12	0.960
90_12_24	303	25	-8	0.960
90_12_48	314	16	-5	0.960
NC	330	13		0.590
PC	136	4		

Table 3E PHB4 BMP results (mL CH_4/g ThOD), percentage of untreated control (NC), and Mann-Whitney test p value table for PHB4 thermal pretreatment (**A**) and thermal alkaline pretreatment (**B**); thermal pretreatment BMP ID denotes "pretreatment temperature (°C) pretreatment duration (h)"; thermal alkaline pretreatment BMP ID denotes "pretreatment temperature (°C)_pH_pretreatment duration (h)"; glucose positive control (PC).

Statistically Significant Difference < 0.05

Maximum BMP

A

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
35_3	-2	7	-121	0.985
35_24	5	15	-59	0.500
35_48	-13	21	-219	0.977
55_3	0	12	-99	0.905
55_24	11	8	-3	0.331
55_48	3	7	-70	0.960
90_3	13	3	20	0.253
90_24	7	3	-35	0.960
90_48	**20**	**11**	**86**	**0.191**
NC	11	3		0.590
PC	310	21		

B

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
55_8_3	317	26	0	0.331
55_8_24	349	8	10	0.040
55_8_48	327	16	4	0.134
55_10_3	338	42	7	0.331
55_10_24	329	11	4	0.040
55_10_48	348	14	10	0.040
55_12_3	323	17	2	0.331
55_12_24	**357**	**16**	**13**	**0.040**
55_12_48	326	14	3	0.253
90_8_3	293	42	-7	0.809
90_8_24	322	37	2	0.331
90_8_48	302	38	-5	0.809
90_10_3	356	23	13	0.040
90_10_24	308	23	-3	0.809
90_10_48	327	15	3	0.134
90_12_3	338	51	7	0.331
90_12_24	318	12	1	0.331
90_12_48	252	30	-20	0.985
NC	316	2		0.590
PC	194	8		

Table 3F PLA BMP results (mL CH_4/g ThOD), percentage of untreated control (NC), and Mann-Whitney test p value table for PLA thermal pretreatment (**A**) and thermal alkaline pretreatment (**B**); thermal pretreatment BMP ID denotes "pretreatment temperature (°C) pretreatment duration (h)"; thermal alkaline pretreatment BMP ID denotes "pretreatment temperature (°C)_pH_pretreatment duration (h)"; glucose positive control (PC).

Statistically Significant Difference < 0.05

Maximum BMP

A

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
35_3	10	10	401	0.038
35_24	3	2	191	0.038
35_48	2	3	164	0.038
55_3	8	7	328	0.036
55_24	1	1	140	0.036
55_48	4	3	215	0.038
90_3	0	4	108	0.058
90_24	26	2	852	0.036
90_48	**63**	**16**	**1943**	**0.038**
NC	-3	0		0.604
PC	13	2		

B

BMP ID	40 Day BMP	Stdev	% diff NC	p-value
55_8_3	7	6	438	0.092
55_8_24	6	3	354	0.058
55_8_48	9	1	552	0.038
55_10_3	9	1	595	0.036
55_10_24	9	2	541	0.038
55_10_48	42	47	2994	0.038
55_12_3	16	4	1083	0.036
55_12_24	21	29	1465	0.092
55_12_48	5	1	279	0.036
90_8_3	9	3	543	0.038
90_8_24	50	1	3557	0.036
90_8_48	84	4	6107	0.038
90_10_3	13	9	862	0.036
90_10_24	50	4	3561	0.038
90_10_48	**86**	**9**	**6237**	**0.038**
90_12_3	43	45	3074	0.038
90_12_24	46	2	3278	0.038
90_12_48	84	3	6060	0.038
NC	1	1		0.604
PC	172	2		

Appendix 3 References

Speece, R.E., 2008. Anaerobic Biotechnology and Odor/Corrosion Control for Municipalities and Industries. Archae Press, Nashville, Tenn

Appendix 4

The appendix to “4 METHANE YIELD and LAG CORRELATE with BACTERIAL COMMUNITY SHIFT FOLLOWING PHB BIOPLASTIC ANAEROBIC CO-DIGESTION” contains the following tables and figures: Co-digestion functional meta data **(Table 4A)**, ANOSIM data values **(Table 4B)**, Alpha diversity indices **(Figure 4A)**, Major bacterial and archaeal NMDS plots **(Figure 4B)**, and Heatmap of 30 significant OTUs **(Figure 4C)**

Table 4A Co-digestion functional meta data for pre- and post-co-digestion.

	Pre-co-digestion Period				Post-co-digestion Period			
	PHB1 Untreated	**PHB1 Pretreated**	**PHB2 Untreated**	**PHB2 Pretreated**	**PHB1 Untreated**	**PHB1 Pretreated**	**PHB2 Untreated**	**PHB2 Pretreated**
Biogas (L/LR-Day)	2.9 ± 0.3	2.8 ± 0.3	2.8 ± 0.5	2.9 ± 0.3	3.5 ± 0.3	3.4 ± 0.4	3.4 ± 0.3	3.3 ± 0.2
CH_4 (L/LR-Day)	1.9 ± 0.02	1.9 ± 0.02	1.9 ± 0.04	1.9 ± 0.01	2.3 ± 0.01	2.3 ± 0.01	2.2 ± 0.01	2.2 ± 0.02
CH_4 (%)	67 ± 3	67 ± 4	68 ± 4	67 ± 4	65 ± 0.4	64 ± 0.7	65 ± 0.4	66 ± 0.6
Lag phase (Days)	NA	NA	NA	NA	11.5 ± 0.7	6.8 ± 4.2	6.0 ± 1.4	2.5 ± 0.7
pH	7.3 ± 0.02	7.3 ± 0.03	7.3 ± 0.02	7.3 ± 0.02	7.3 ± 0.05	7.3 ± 0.05	7.3 ± 0.04	7.3 ± 0.04
VFA (mg/L)	47 ± 3	51 ± 6	48 ± 5	46 ± 2	47 ± 4	47 ± 4	45 ± 2	45 ± 3
VS Reduction (%)	77 ± 1	76 ± 2	77 ± 1	76 ± 1	81 ± 1	78 ± 1	78 ± 1	78 ± 1

Table 4B ANOSIM data values corresponding to Figure 4.

(A)		Pre-co-digestion Period vs Transition Period	Transition Period vs Post-co-digestion Period
Major Bacterial OTUs	PHB1-Untreated	R=0.509, P = 0.002	R=0.678, P = 0.001
	PHB1-Pretreated	R=0.815, P = 0.006	R=0.935, P = 0.001
	PHB2-Untreated	R=0.765, P = 0.002	R=0.996, P = 0.004
	PHB2-Pretreated	R=0.519 P = 0.004	R=0.535, P = 0.001
(B)			
30 Significant Bacterial OTUs (Spearman)	PHB1-Untreated	R=0.79, P = 0.01	R=0.75, P = 0.003
	PHB1-Pretreated	R=0.97, P = 0.007	R=0.65, P = 0.002
	PHB2-Untreated	R=0.90, P = 0.002	R=0.84, P = 0.003
	PHB2-Pretreated	R=0.92, P = 0.004	R=0.45, P = 0.004

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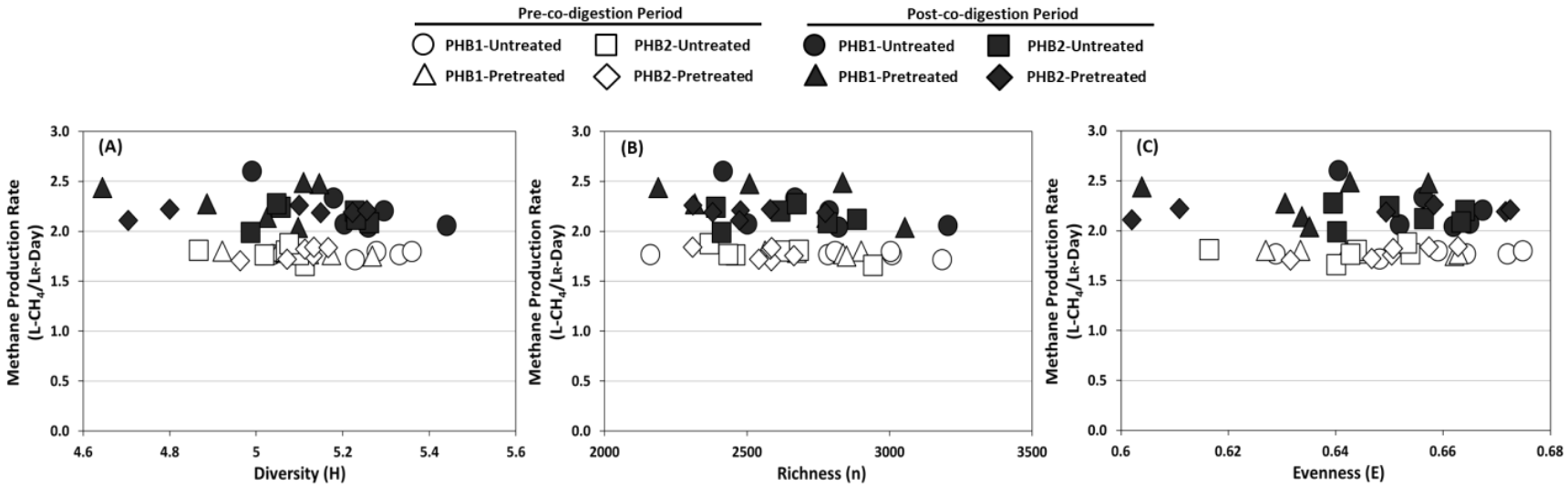

Figure 4A Alpha diversity indices versus methane production rate employing all 10,675 OTUs for pre- and post-co-digestion periods, including (A) Shannon-Weaver diversity index (H), (B) OTU richness, and (C) evenness.

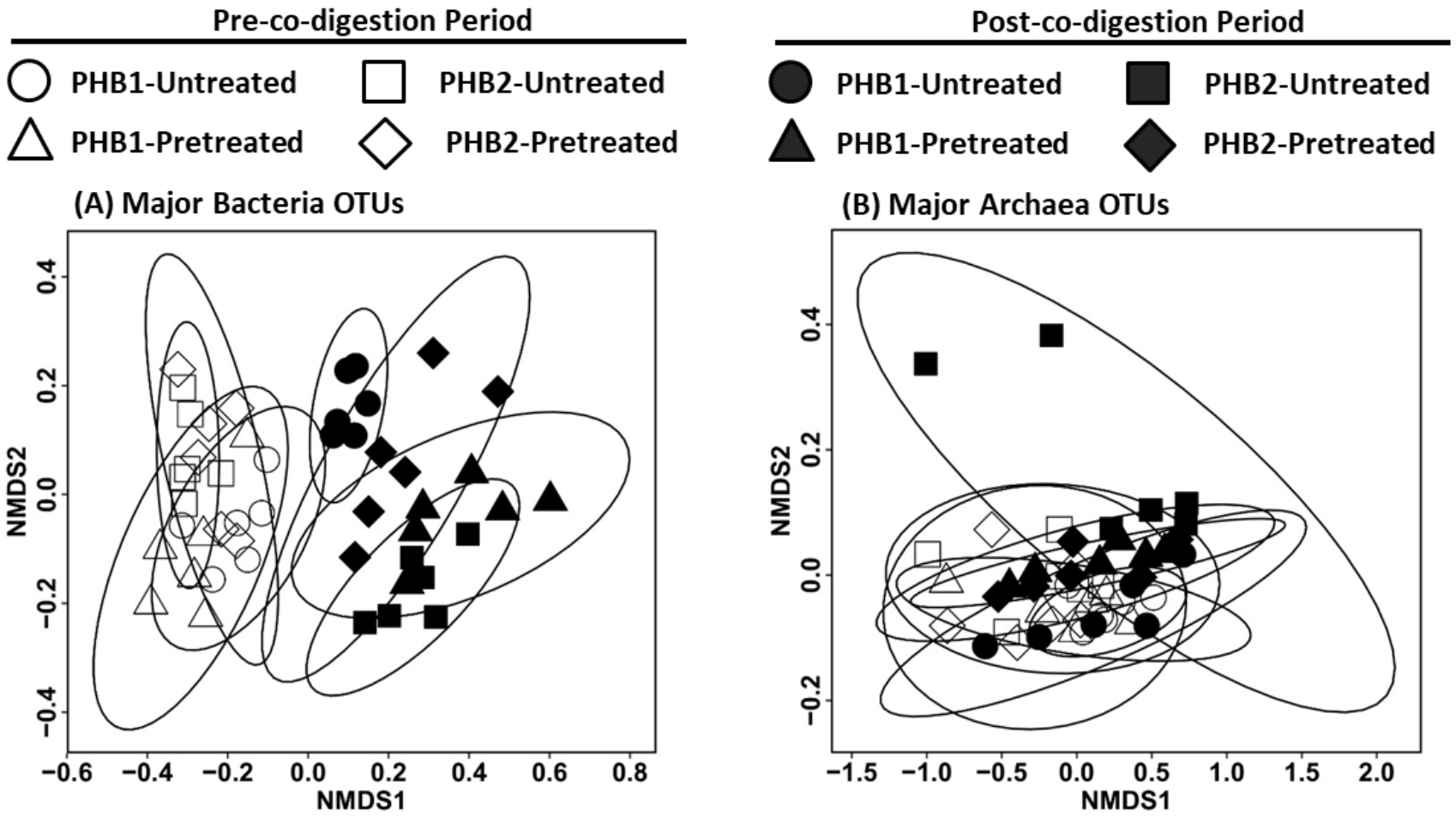

Figure 4B Major (A) bacterial OTUs and (B) archaeal OTUs community comparison during pre- and post-co-digestion period NMDS plots (i.e., > 0.1% relative abundance in at least one sample).

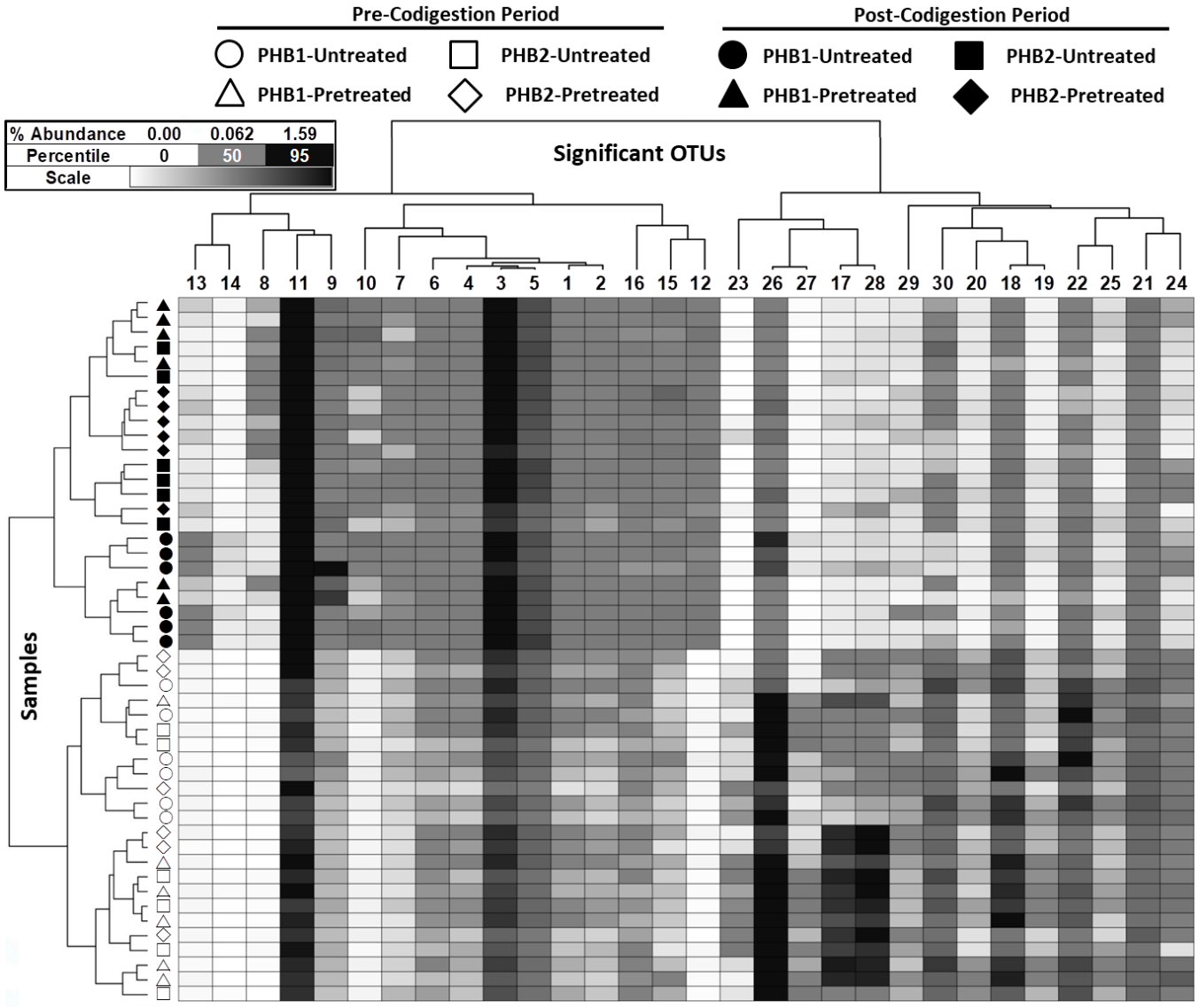

Figure 4C Relative abundance heatmap with dual hierarchical clustering of 30 significant OTUs identified through Spearman correlation with methane production from pre- and post-co-digestion samples based upon Bray-Curtis distances using Spearman correlation again to cluster both OTUs and Samples. Samples cluster into two branches with post-co-digestion samples on top and pre-co-digestion on bottom. Significant OTUs cluster into positively correlated OTUs on the left and negatively correlated on the right