Research Article

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Toward sustainable dairy waste utilization: enhanced VFA and biogas synthesis via upcycling algal biomass cultured on waste effluent

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Abstract

BACKGROUND: In 2012, 9.3 million head of dairy cows in the USA produced an estimated 20 million metric tons of manure solids, but little value was gained from this manure. There is a pressing need to enhance manure resource recovery efforts, as dairy manure has potentially significant environmental impacts. This study evaluated components of an integrated suite of biological processes designed to maximize resource recovery from dairy manure, in which algae grown on polyhydroxyalkanoate (PHA) production effluent (PHA-algae) were fermented and anaerobically digested to determine process impacts.

RESULTS: A 10% PHA-algae supplement produced 11% more volatile fatty acids (VFA) during fermentation and 11% more methane during anaerobic digestion (AD) (vs. dairy manure); the PHA-algae biogas also contained a higher percentage (62.7 vs. 59.1%) of methane than manure biogas. Algal augmentation exhibited no negative effect on fermenter or AD operation. Quantitative polymerase chain reaction (PCR) showed that the ADs contained substantial populations of both acetoclastic and hydrogenotrophic methanogens, which, given the heterogeneous substrate, enhanced process stability. There were significant differences between PHA-algae batches, and large quantities of COD were released during algae freezing.

CONCLUSION: PHA-algae yielded more VFA during fermentation, and a more methane-rich biogas following AD than dairy manure. A 10% PHA-algae supplement caused no process disturbance in normal manure flora.

Keywords: algae; anaerobic digestion; fermentation; polyhydroxyalkanoate (PHA); volatile solids

INTRODUCTION

In 2012, 9.3 million head of dairy cows in the USA (NASS, 2014) produced 91 million metric tons of milk;[1] concurrently, dairy operations generated an estimated 20 million metric tons of manure solids,[2] and while milk sales were valued at $35.3B,[3] very little value was gained from the manure. Specifically, less than 4% of the manure solids were processed through anaerobic digestion (AD) for resource recovery .[4] There are several reasons why much of the dairy manure resources remain untapped. For instance, anaerobic digesters are capital intensive, resource recovery operations can distract from dairy operations, and AD biogas cannot compete with the price of natural gas which decreased 40% from 2008 to 2014.[5]

While manure resource recovery efforts have been limited to date, there is nonetheless a pressing need to enhance these efforts in the future, as dairy manure processing has potentially significant environmental impacts. In 2011, greenhouse gas (GHG) emissions from the agricultural sector constituted an estimated 6.9% of the USA GHGs, 7.0% of which were from dairy manure management.[6] Manure nutrient management is also a challenge, as each ton of manure contains approximately 6.6 kg of nitrogen (N) and 1.1 kg of phosphorus (P);[7] improper management of manure nutrients can potentially impair receiving water quality.

Manure-derived polyhydroxyalkanoate (PHA), which is a high-value biodegradable thermoplastic, has been suggested as a commodity that could improve the manure resource recovery footprint over biogas production alone (both economically and environmentally).[8] PHA can be synthesized from volatile fatty acids (VFAs) produced in a short retention time fermenter without compromising AD operations .[9,10] Life-cycle assessment analysis of

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PHA production also shows that two-stage dairy waste processing can reduce GHG production by up to 60%.[8] PHA is a valuable product by itself,[11] is easily stored and transported, and retains almost all of the carbon of the original feedstock, and finally, PHA can be produced using mixed microbial cultures.[12] Nevertheless, while PHA production addresses many operational and environmental concerns of manure resource recovery, PHA bioreactors remove little N or P. Therefore, PHA production and AD must be coupled with nutrient capture technologies, such as algal production.

Co-fermentation/co-digestion of algal biomass with dairy manure is topical, and several researchers have addressed benefits of combining dairy manure with algae cultivation in order to capture nutrients.[13–15] Our research team has investigated algal growth on manure-derived wastewater, as a means to sequester additional N and P,[16,17] and is further interested in algal biomass recycling as a means to enhance AD. Algae and manure wastes might be expected to co-digest well, as both contain residual products of plant metabolisms; however, there are differences between the two substrates. One of the challenges of integrating algae, PHA production, and dairy manure processing is that the sources of process variability must be better understood and quantified in order to provide optimal process control. In particular related to AD, algae appears potentially problematic because a wide range of methane yields have been reported for algae AD, ranging from 0.09 up to 0.45 LCH_4 $g^{-1}VS$.[18] Several reasons are thought to account for the differences in methane yields, such as changes in algal composition, conversion conditions, AD inhibition, and algal pretreatment effects,[18] but any additional sources of process variation do need to be identified in order to achieve optimal process integration.

Several of the individual resource recovery concepts discussed above have been investigated independently, but a more detailed assessment of the influence of incorporating algal biomass into a manure–PHA resource recovery framework has not been reported. Thus, the purpose of this research was to determine the feasibility and relative productivity of algae grown on PHA-process effluent (PHA-algae). Specific objectives were to: (i) assess the potential to increase VFA production and/or effect speciation in a fermenter primarily fed manure; (ii) assess the potential to increase AD biogas yield and/or specific methane content; and (iii) identify potential explanations for some of the methane yield disparities in the literature.

MATERIALS AND METHODS

Source and characteristics of dairy manure and algae

Raw dairy manure was obtained from the University of Idaho (100–120 head dairy farm); fresh manure was collected on a semi-weekly basis and stored at 4 °C until use.

Algae (*Chlorella vulagris* UTEX#2714) grown on 10% PHA bioreactor effluent (provided by the Coats lab, Dept. of Civil Engineering, University of Idaho, Moscow, ID) and 90% municipal water was supplied by the Dept. of Biological Science, Boise State University, Boise, ID. PHA-algal biomass was cultivated, in triplicate, using 200 L pilot-scale raceway ponds (140 cm × 60 cm × 20 cm) each with a working volume of 110 L under semi-continuous operation after stationary phase was established. Raceways were operated continuously for 30 days with an average 4 day hydraulic retention time (HRT). Low shear circulation was provided by submersible water fans (Marine Depot, Hydor Koralina) with a mixing rate of ~1.21 L/s. Each pond was equipped with a separate data logging Arduino control board (AdaFruit, Arduino Mega ATmega2560) using LabView software to monitor and record various inputs into the system. Arduino compatible sensors were used to collect data on temperature (Atlas Scientific, ENV-TMP), pH (Atlas Scientific pH Sensor), PAR irradiance (Apogee Instruments SQ-222) and CO_2 injection volume (Atlas Scientific, TurboFlow-226000). Environmental cultivation conditions included an 18:6 light:dark photoperiod provided by direct sunlight (~2000 μmol photons m^{-2} s^{-1}) which was supplemented by high pressure metal halide lights (250 μmol photons m^{-2} s^{-1}) during low light conditions (e.g. early morning, late evening, and cloudy conditions). Temperature was controlled at 25 °C using submersible heaters (Eheim Jager Thermostat, 250 W heater). The pH was regulated at 6.5 ± 0.05 using CO_2 sparged into the culture in response to increases in pH. Based on an initial percentage volatile solids (VS) measurement, quantities of algae for the fermentation and digestion tests were weighed and maintained at −20 °C until needed.

Manure and algae fermentation

Three 1 L completely-mixed fermenters (designated '0', '1', and '2') were incubated in an enclosed shaker table at room temperature (mean of 26.4 °C ±0.6 °C) with target organic loading rates (OLRs) of 9.4 gVS day^{-1}, and were decanted and fed daily to achieve a 4-day HRT. All three fermenters were fed manure for 32 days to achieve operational stability and to establish baseline operations before augmentation with algae; thereafter, experimental fermenter '1' received 90% manure and 10% PHA-algae (VS weight basis) for 72 days, while fermenters '0' and '2' remained as manure-fed controls. A 10% VS algal loading was selected as a practical compromise between algae availability and expected detectability of experimental effects. Fermenter influent, and effluent total solids (TS), VS, and VFA were measured daily.

Manure and PHA-algae AD

Three 2 L completely-mixed ADs were incubated in an enclosed shaker table (mean temperature of 37.7 °C ±0.4 °C), and each AD was decanted and fed concentrated fermenter effluent daily to achieve a 20 day HRT. Concentrated fermenter effluent was prepared by centrifugation (10 000 rpm = 17 700 × G, for >5 min), with the resulting pellet re-suspended in supernatant to achieve a target AD OLR. All three digesters received fermented manure for 32 days to achieve operational stability and establish baseline operations, thereafter, digester '0' was maintained as the manure control, digester '1' received fermented manure and fermented algae, and digester '2' received fermented manure and raw algae. Each day, 5.9 gVS from fermenters '0' and '1' were fed to digesters '0' and '1', respectively, while AD '2' received 5.0 gVS from fermenter '2' and 0.9 gVS raw PHA-algae.

Biogas from each digester was collected in gas accumulators and daily biogas volumes were measured by water displacement. Gas samples from the accumulator were tested for methane, carbon dioxide, and nitrogen. Methane production volumes were reported as methane at STP, based on the total gas volume, methane composition, local atmospheric pressure, and temperature.

Analytical techniques

Percentage TS and VS were measured in accordance with Standard Methods.[19] Samples were centrifuged and filtered (0.22 μm PVDF syringe filters) prior to testing for soluble constituents. pH was measured using an Accumet AP85 pH meter (Thermo-Fisher Scientific Corp, Waltham, MA, USA).

Table 1. Primer sequences and annealing temperatures for qPCR

Target group	Sequence	Anneal-ing temp (°C)	16S rDNA copy No.†
Methanococcales (MCC 495 F)*	TAA GGG CTG GGC AAG T	59	2.86
Methanococcales (MCC 832 R)*	CAC CTA GTY CGC ARA GTT TA	59	2.86
Methanobacteriales (MBT 857 F)*	CGW AGG GAA GCT GTT AAG T	59	2.5
Methanobacteriales (MBT 1196 R)*	TAC CGT CGT CCA CTC CTT	59	2.5
Methanomicrobiales (MMB 282 F)*	ATC GRT ACG GGT TGT GGG	59	2.25
Methanomicrobiales (MMB 832 R)*	CAC CTA ACG CRC ATH GTT TAC	59	2.25
Methanosarcinales (MSL 812 F)*	GTA AAC GAT RYT CGC TAG GT	59	3.0
Methanosarcinales (MSL 1159 R)*	GGT CCC CAC AGW GTA CC	59	3.0
Methanosarcinaceae (Msc 380 F)*	GAA ACC GYG ATA AGG GGA	56	3.0
Methanosarcinaceae (Msc 828 R)*	TAG CGA RCA TCG TTT ACG	56	3.0
Methanosaetaceae (Mst 702 F)*	TAA TCC TYG ARG GAC CAC CA	59	2.0
Methanosaetaceae (Mst 862 R)*	CCT ACG GCA CCR ACM AC	59	2.0
Archaea (ARC 787 F)*	ATT AGA TAC CCS BGT AGT CC	56	1.8
Archaea (ARC 1059 R)*	GCC ATG CAC CWC CTC T	56	1.8
Bacteria (BAC 338 F)**	ACT CCT ACG GGA GGC AGC AG	60	4.1
Bacteria (BAC 533 R)**	TTA CCG CGG CTG CTG GCA C	60	4.1
Prokaryotes (PRK 341 F)*	CCT ACG GGR BGC ASC AG	55	4.1
Prokaryotes (PRK 806 R)*	GGA CTA CYV GGG TAT CTA AT	55	4.1

†Chosen as representative of the taxon
*Ref. 37
**Ref. 38

VFA concentrations were measured as described elsewhere.[10] VFA were quantified separately as acetic (H-Ac), propionic (H-Pr), butyric (H-Bu), isobutyric (H-iBu), valeric (H-Va), isovaleric (H-iVa), and caproic (H-Ca) acids using linear standard curves, and masses were converted to COD equivalents using direct stoichiometric ratios. Lactic/formic acid and H-Ac were quantified as described elsewhere.[20] Formic and lactic acids co-eluted so data were presented as lactic acid equivalents. Theoretical estimates of VFA vapor pressures pre- and post-drying were based on fitting Antoine parameters to the reported vapor pressures.[21]

Biogas composition was measured as described elsewhere.[10] Methane, carbon dioxide, and nitrogen volumes were determined by matching with standard curves, setting the biogas water vapor saturation to that of room temperature (~3% by volume), and converting volumes to STP.

Microbial population analyses

Genomic DNA was extracted from biomass obtained from each AD on six different days during the 104 day AD operational period, with two samples before, and four samples after algae addition (~2.0 mL samples). Quantitative real-time PCR (qPCR) was applied using 16S rDNA-based oligonucleotide primers (Table 1) as published elsewhere[10] to estimate the relative abundance of the respective archaeal populations present in the ADs. Three Orders of hydrogenotrophic methanogens (*Methanococcales*, *Methanobacteriales*, and *Methanomicrobiales*), and the two most predominant families within *Methanosarcinales* (*Methanosarcinaceae* and *Methanosaetaceae*) were normalized as a fraction of the total archaeal population.[10] Relative expression ratio (RER) was determined using the approach of Čikoš and Koppel.[22]

Statistical methods

Paired student *t*-tests were used for statistical comparisons and differences were declared significant at $P < 0.05$. Tests for normality used the extended Shapiro–Wilk test,[23] and both normal and log-normal data distributions were evaluated by graphical inspection[24] of untransformed and log transformed data, respectively. Chauvenet's criterion[25] was used to identify and eliminate outliers.

Chemical speciation screening

Mineral speciation testing used Visual Minteq[26] to quantify the buffering effect of manure minerals. Published manure mineral composition[27] were weighed by dry mass and used with the measured VFA spectrum and 0.9 Atm CO_2, to compare the buffering effect of 100% manure solids with 90% manure solids.

Impact of algae sample freezing

Three batches of algae were subject to 0, 1, 2, 3, and 5 freezing and thawing cycles. Fresh algae were diluted in 4 volumes of deionized water, 5 mL volumes placed in re-sealable (Ziploc®) freezer bags, frozen (<20 °C for 1 h) then thawed (room temperature). Samples were removed after the appropriate freeze/thaw cycle number and centrifuged (12 500 rpm for 10 min) before filtering (0.22 µm PVDF syringe filters) the supernatant, and testing duplicates for soluble COD (sCOD). Results were standardized on the first batch %VS.

RESULTS AND DISCUSSION

Feedstock characterization and pre-investigation bioreactor stabilization

Manure and PHA-algae characteristics, assessed based on TS and VS, were quite consistent during the experimental period (Table 2). The %VS for the respective substrates was typical for organic matter, confirming the bio-conversion potential for these investigations. For the algal-supplemented investigations, potential loading variability was reduced by regularly adjusting manure and algae feed quantities based on initial VS determinations.

Table 2. Characteristics of dairy manure, and algae batches grown on PHA-processing wastes

Material	Percentage total solids	Percentage volatile solids
Raw dairy manure	14.57 ± 1.76 (298)†	84.17 ± 1.49 (298)
Algae	16.55 ± 2.37 (15)	90.65 ± 0.96 (15)

†Mean ± standard deviation (number of samples analyzed)

Stable fermenter and AD operation (prior to beginning augmentation of algal biomass) was evaluated for 32 days by feeding only manure at 4- and 20-days HRT, yielding stabilization periods of 8- and 1.5-HRTs for the fermenters and ADs, respectively. If AD stabilization periods are too short, VFA accumulate because the stressed methanogens fail to remove sufficient VFA. In this case the 1.5-HRT AD stabilization period was confirmed sufficient, because AD VFA consumption was generally complete, with only the occasional trace quantity of VFA present in the AD effluent after the first week of operation. Results were also consistent with our prior investigations.[10,28]

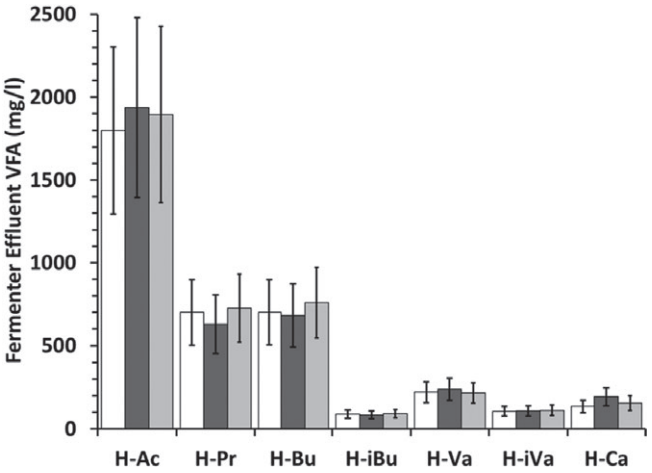

Figure 1. Fermenter effluent VFA concentrations. VFA concentrations are shown on the Y-axis while the X-axis shows acetic (H-Ac), propionic (H-Pr), butyric (H-Bu), isobutyric (H-iBu), valeric (H-Va), isovaleric (H-iVa), and caproic (H-Ca) acids in mg L^{-1}. White and light grey are control (fed 100% manure VS) fermenters '0' and '2', respectively. Dark gray is experimental fermenter '1' (fed 90% manure and 10% algae VS grown on PHA process wastes). Error bars indicate ± standard deviation.

Fermenter performance

Fermenter feed changes can affect the quantity and type of VFAs produced, however, no substantial changes were observed in this case (Table 3), confirming the relative consistency of the substrates. Comparing fermenter performance once algal augmentation commenced, there were only three notable performance differences observed between fermenter 1 (manure + algae) and the two manure controls (Table 3). While the effluent pH was 0.14 lower in fermenter 1, which was of no real consequence, VFA yield and speciation appeared affected by algal augmentation. Most interestingly the mean VFA yield of PHA-algae was 11.5% higher than manure (as gVFA g^{-1}VS applied). Assuming the manure conversion within fermenter 1 was similar to the controls, 0.654 g of the daily 0.732 gVFA yield was derived from manure (Table 3), with the difference from the algae VS, giving a VFA yield of 86.1 mgVFA g^{-1}algae VS applied (Table 3). The manure VFA yield of ~0.47 gVFA g^{-1}VS removed (Table 3) were higher than for batch reactors reported previously (0.35 to 0.43 gVFA g^{-1}VS removed[28]); this might be because this study used fed-batch systems that experience more gradual changes in substrate load, which allows acclimation and adaptation of the biomass.

Regarding VFA speciation, there were statistically significant differences in effluent VFA composition between the control and experimental fermenters; fermenter 1 had 10% lower H-Pr, 10% higher H-Va, and 30% higher H-Ca than the control fermenters (Figure 1). High hydrogen partial pressures can thermodynamically favor the accumulation of H-Ca and H-Va; however, the potential effect of hydrogen accumulation is not entirely clear because

Table 3. Fermenter performance at 4-day HRT and ~26 °C incubation

Feed composition	Fermenter 0 Manure control	Fermenter 1 Manure:algae as 9:1 VS	Fermenter 2 Manure control
Manure influent (gVS day^{-1})	9.386 ± 0.503 (70)†	8.473 ± 0.466 (70)	9.384 ± 0.504 (70)
Algae influent (gVS day^{-1})	-	0.901 ± 0.076 (69)	-
Fermenter effluent (gVS day^{-1})	7.869 ± 0.555 (67)	7.828 ± 0.517 (67)	7.791 ± 0.682 (67)
VS reduction (gVS day^{-1})	1.506 ± 0.69 (68)	1.562 ± 0.688 (67)	1.631 ± 0.866 (68)
VS conversion (%)	15.9 ± 6.9 (68)	16.4 ± 6.9 (67)	17.2 ± 8.8 (68)
Effluent pH	6.23 ± 0.19 (23)	6.08 ± 0.16 (20)	6.21 ± 0.14 (20)
VFA yield (gVFA day^{-1})	0.697 ± 0.164 (53)	0.732 ± 0.159 (53)	0.752 ± 0.191 (55)
VFA yield (mgVFA g^{-1}VS applied)	74.7 ± 18.1 (53)	78.5 ± 17.8 (53)	78.4 ± 24.4 (57)
VFA yield (gCOD day^{-1})	1.227	1.199	1.302
VFA yield (gVFA g^{-1} VS removed)	0.455 ± 0.245 (50)	0.474 ± 0.253 (53)	0.493 ± 0.272 (52)
Influent VFA (gVFA day^{-1})	0.204 ± 0.121 (58)	0.205 ± 0.122 (58)	0.204 ± 0.121 (58)
Influent VFA (gCOD day^{-1})	0.264 ± 0.16 (58)	0.265 ± 0.161 (58)	0.264 ± 0.16 (58)
Effluent VFA (gVFA day^{-1})	0.969 ± 0.243 (49)	0.954 ± 0.243 (54)	1.018 ± 0.25 (50)
Effluent VFA (gCOD day^{-1})	1.387 ± 0.351 (49)	1.36 ± 0.349 (54)	1.462 ± 0.36 (50)
VFA from manure (mgVFA g^{-1}VS applied)		77.2	
VFA from algae (mgVFA g^{-1}VS applied)		86.1	

†Mean ± Standard deviation (number of samples)

fermenter 1 also produced higher levels of H-Ac and lower levels of H-Pr and H-Bu than the control fermenters, a pattern normally associated with lower hydrogen partial pressures. The higher H-Ac of fermenter 1 is more likely a by-product of algae lipids degraded using metabolic pathways such as the beta-oxidation pathway that preferentially increase the acetate concentration. Regardless of the cause, the production of longer chain VFAs could enhance PHA production, as this substrate is used by bacteria to produce longer chain hydroxyalkanoic acids that ultimately enhance polymer characteristics.[29] Ultimately, the 11.5% larger VFA yield from PHA-algae (Table 3) coupled with the more diverse VFA speciation confirms that PHA-algae will be a suitable feedstock for VFA production. Furthermore, the results suggest that a full-scale PHA-algae fermenter will be operationally stable, not easily overloaded, and will not require sensitive controls.

Regarding the observed pH difference in Fermenter 1 vs. the control fermenters, the effluent pH decrease of 0.14 was statistically and also chemically significant, because the substantial alkalinity present in dairy manure means that large composition changes are required to effect even small pH changes. Formic- and lactic-acid concentrations were tested to determine whether these might account for the observed pH difference, but neither was present in significant concentrations (Table 4). However, when the mineral buffering of dairy manure was tested computationally (Minteq) the control fermenter pH was found to be 6.136 and close to the measured pH 6.22 (Table 3). In contrast, fermenter 1 with its more 'dilute' manure mineral components had a Minteq pH of 6.014, close to the measured pH 6.08, and as the computational pH difference accounted for 87% of the observed pH difference, mineral buffering dilution was concluded to be the most likely explanation for the observed fermenter pH difference.

Table 4. Lactic (+ formic) acid concentrations

Sample source	Lactic*Acid (mg L^{-1})
Dairy manure	65 ± 89 (7)†
Control fermenters (0 and 2) effluent – fed manure	7 ± 18 (7)
Fermenter (1) effluent – fed raw algae & manure	14 ± 24 (7)
AD(0) effluent – fed fermented manure	10 ± 22 (7)
AD(1) effluent – fed fermented algae & manure	7 ± 16 (7)
AD(2) effluent – fed raw algae & fermented manure	10 ± 19 (7)

*Lactic- and formic-acid co-elute in this HPLC test. Lactic acid values represent maximum concentrations.
†Mean ± Standard deviation (number of samples analyzed).

Anaerobic digester (AD) performance

AD performance was stable over the full assessment period, with only trace VFA amounts detected in the effluent during both the initial stabilization and experimental periods. AD results were consistent with our previous pre-fermented manure investigations,[10] and biomethane production was not impaired by the nitrogen-rich manure. While mean manure VS loading to AD2 (manure + raw algae) was 5.3 gVS day^{-1} (Table 5) and slightly higher than the 5.0 gVS day^{-1} target, as the combined manure + algae VS overloading was only 5% higher than the AD0 and AD1 loading, it is not thought to have had a significant impact on the results. When the manure methane contribution (AD0) was subtracted from the AD2 methane yield, the PHA-algae yield (mL CH_4 g^{-1} VS) was estimated to be 11.2% higher than dairy manure (Table 5). The enhanced productivity of algae was similar to the phenomenon observed in the fermenters.

Experimental and control ADs produced similar methane quantities (that were also generally consistent with other algal-based AD systems,[30] and not specific to the algal species used herein), but the methane *concentration* of the PHA-algae biogas systems (AD1 and AD2) was statistically significantly higher (Table 5). Further interrogation of the data, however, suggests that the increased methane concentration appeared to be due to an unexplained decrease in CO_2 production, and not from actual increased relative methane production. Theoretically, AD0, AD1, and AD2 (Table 5) would have produced daily biogas volumes of 1826, 1674, and 1915 mL, respectively, if all the biogas had the methane concentrations of AD0. However, as AD1 and AD2 actually produced 1577 and 1806 mL biogas (at 62.7% CH_4 content), it appears that 97 and 110 mL of CO_2 were 'missing' from the biogas of AD1 and AD2,

Table 5. AD performance at 20-day HRT and 37.7 °C incubation

Substrate - fermented unless stated	AD 0 Manure (control)	AD 1 Manure + algae	AD 2 Manure + raw algae
Influent manure (gVS day^{-1})	5.91 ± 0.51 (63)†	5.9 ± 0.43 (65)	5.31 ± 0.49 (65)
Algae influent (gVS day^{-1})	–	–	0.91 ± 0.08 (70)
AD effluent (gVS day^{-1})	3.95 ± 0.32 (66)	3.76 ± 0.32 (69)	3.89 ± 0.38 (67)
VS reduction (g)	1.99 ± 0.68 (64)	2.14 ± 0.5 (65)	2.34 ± 0.54 (63)
VS conversion (%)	33.4 ± 7.8 (61)	36.7 ± 6.2 (64)	37.3 ± 7.1 (63)
pH	7.49 ± 0.09 (29)	7.51 ± 0.1 (26)	7.52 ± 0.07 (27)
VFA influent (gVFA day^{-1})	0.35 ± 0.09 (56)	0.37 ± 0.07 (53)	0.35 ± 0.07 (55)
VFA influent (gCOD day^{-1})	0.53 ± 0.13 (48)	0.51 ± 0.1 (54)	0.52 ± 0.1 (50)
CH_4 (mL day^{-1})	1079 ± 126 (61)	989 ± 105 (59)	1131 ± 108 (58)
CH_4 (mL g^{-1}VS applied)	179 ± 19 (56)	170 ± 22 (57)	184 ± 16 (51)
CH_4 Yield (mL g^{-1}VS removed)	549 ± 153 (54)	468 ± 100 (53)	489 ± 113 (51)
CH_4 Fraction (%)	59.1 ± 5.3 (32)	62.7 ± 2.2 (27)	62.7 ± 2.1 (26)
CH_4 from manure (mL g^{-1}VS applied)			179
CH_4 from algae (mL g^{-1}VS applied)			200

†Mean ± Standard deviation (number of samples analyzed)

Table 6. AD qPCR Relative Expression Ratio (RER) from algae supplementation period, and methanogenic proportion of archaea

	RER between digesters†			Methanogens as % of Archaea†		
Orders, families, and domains	AD1 to AD0	AD2 to AD0	AD2 to AD1	AD0	AD1	AD2
Methanococcales	1.25 ± 0.91	0.72 ± 0.42	0.62 ± 0.27	2.2 ± 0.8	2.5 ± 1.1	2.2 ± 0.6
Methanobacteriales	1.25 ± 0.83	0.94 ± 0.64	0.74 ± 0.09	11.0 ± 8.3	12.2 ± 8.0	8.6 ± 4.2
Methanomicrobiales	0.73 ± 0.49	0.88 ± 0.70	1.18 ± 0.45	35.8 ± 5.0	35 ± 9.5	38.6 ± 9.4
Methanosarcinales	0.74 ± 0.11	0.68 ± 0.39	0.97 ± 0.65	38.2 ± 24.6	37.4 ± 22.6	27.9 ± 13.5
Methanosarcinaceae	2.17 ± 1.89	0.86 ± 0.72	0.39 ± 0.06	19.3 ± 14.0	24.6 ± 12.4	18.1 ± 9.5
Methanosaetaceae	1.47 ± 0.48	0.92 ± 0.70	0.70 ± 0.52	27.3 ± 18.0	29.4 ± 19.7	33.3 ± 21.2
Archaea	1.34 ± 0.65	1.20 ± 1.09	0.78 ± 0.33			
Prokaryotes	1.28 ± 0.48	1.08 ± 0.25	0.99 ± 0.58			
Bacteria	1.92 ± 2.12	0.90 ± 0.59	0.72 ± 0.36			

†Mean ± Standard deviation, with four samples tested

respectively. The 'missing' CO_2 increases methane yields because the digester liquor was probably supersaturated with methane,[31] and the decreased CO_2 volume directly affects the gas transfer coefficient ($K_l a$). When the missing CO_2 gas volume was theoretically restored, the $K_l a$ and transferred gas volumes increased proportionally, and the theoretical methane yields of AD1 and AD2 became 497 and 519 $mLCH_4\ g^{-1}$ S removed, respectively, which are statistically indistinguishable from the AD0 manure control (549 $mLCH_4\ g^{-1}$ VS removed). Similarly, the AD1 and AD2 methane yields would theoretically increase to 181 and 195 $mLCH_4\ g^{-1}$VS applied, which is similar to the AD0 yield 179 $mLCH_4\ g^{-1}$VS applied. These similar theoretical methane production values suggest there was little difference actually realized between the methane yield of the different substrates, and that the biogas methane concentration increase is due to the CO_2 absorbing characteristic of PHA-algae digester liquor. Furthermore, as the 'missing' CO_2 represents about 20% of the CO_2 volume released by the AD control, it is not insignificant.

AD VS removal was ~36% (Table 5) and more than twice that of the fermenters (Table 3); this difference was principally due to the longer retention time and higher temperatures of the AD. Nevertheless, it is not essential that PHA-algae be highly digestible to serve a useful role in a manure/PHA resource recovery system, because there are benefits to algae proteins being recalcitrant to digestion. For instance, if digested algae retain recalcitrant protein, the converted N will be sequestered in a non-inhibitory and readily separable form which offers possibilities as a slow release N fertilizer.[32]

Examining the microbial AD population, while the mean relative expression ratio (RER) did appear to change between the methanogenic taxa in the digesters (Table 6), in fact these patterns were not statistically significant mostly due to the variability in quantified populations between sample dates. There were, however, some consistent patterns; for instance, the main hydrogenotrophic and acetotrophic taxa (*methanomicrobiales* and *methanosarcinales*) were approximately equally represented among the Archaea (Table 6), which suggests that dairy manure-based AD, with and without algal augmentation, may achieve metabolic flexibility in generating methane; similar results were observed with the fermenting bacterial culture in ADs receiving pre-fermented manure.[33] Furthermore, the relative population variability across sampling dates suggest that the ADs might be microbiologically quite heterogeneous, which could further enhance long-term process stability, consistent with previous research[10,33] which demonstrated the microbial robustness of this novel 2-stage AD configuration. Ultimately the augmentation of AD with algal biomass appeared to have no significant effect on the methanogenic population.

Examining the reported differences between fermentation and AD algal methane yields

There are large differences between the methane productivities reported in the peer-reviewed literature for algae AD systems,[30] with the main causes being the large differences in algae composition between species,[34] and the algae preparation and operational conditions.[18] However, several additional potential sources of variability were identified in this investigation that should be considered in the assessment of algal fermentation and AD; these are discussed in the following three sections.

Impact of freezing algae samples

Algae used in these investigations had been frozen, and was thawed before feeding to the fermenter or AD. Freezing may very well enhance the biodegradability of the algae substrate for AD;[30] thus, the effect of freezing was evaluated. Investigations revealed that algae sample storage had a substantial impact on the amount of sCOD released, substrate that would be potentially bio-available for VFA or biogas production. Frozen algae samples released between 43 and 235% more sCOD after a single freeze and thaw cycle (Fig. 2). However, only the first freeze/thaw cycle had this marked effect, as additional freeze/thaw cycles made little difference. Algae samples tested had 93.2%VS (95% confidence limits (CL) of 93.00 to 93.36%VS with n = 3) which is close to, but statistically significantly different from the mean algae 90.7%VS (CL of 90.22 to 91.13%VS and n = 16) used in the experiments. Algae batches A, B, and C were grown under similar conditions yet had an 18.3 g sCOD L^{-1} difference between the highest and lowest sCOD levels after freezing (Fig. 2). An sCOD of 18.3 g L^{-1} is equivalent to ~6 gVS as lipid, or ~18 gVS as VFA; as 37% of the algal VS is anaerobically digestible, the inter-batch sCOD differences appear to range from 9.3 to 28% of the digestible VS, suggesting that accurate algae performance evaluations need to assess several different algae batches and pre-treatment methods even when the algae species is identical, and the culture conditions are similar.

Effect of volatiles loss during solids testing

The effect of VFA-loss was examined because fermenter and AD performance is often reported in units of product *per gVS applied or*

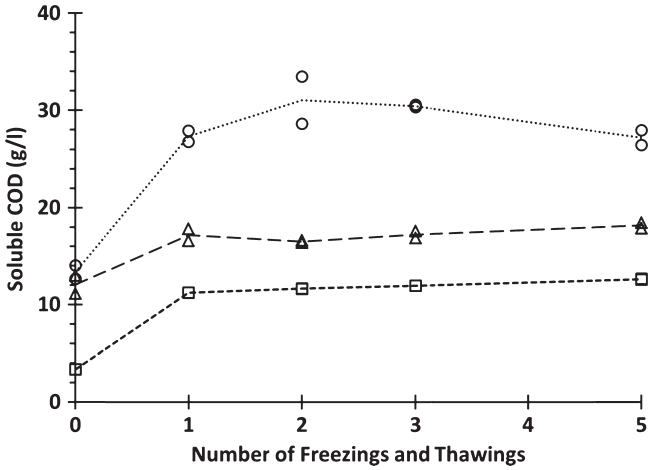

Figure 2. Changes in volatile fatty acid (VFA) concentrations during total solids (TS) drying at 104 °C. Panel A: Y-axis has VFA concentrations after drying, expressed as vapor pressure fraction of the combined VFA vapor pressures, X-axis has VFA vapor pressure fraction before drying. Dotted ovals contain specific VFA with acetic (H-Ac), propionic (H-Pr), butyric (H-Bu), isobutyric (H-iBu), and isovaleric (H-iVa) acids. Panel B has the Panel A data expressed as molarity, and a trend line fitted to the top three VFA (H-Ac, H-Pr, and H-Bu) VFA concentrations.

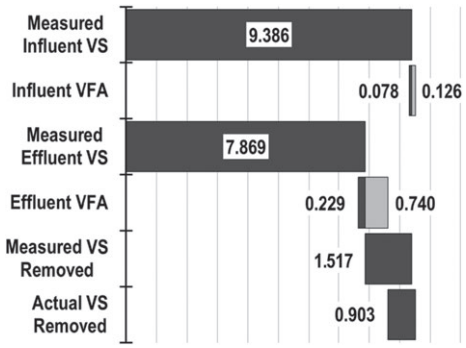

Figure 4. Impact of volatile fatty acids (VFA) loss during volatile solids (VS) testing on the measured control fermenter performance. Dark grey blocks show mass of VS or VFA in grams. Light-grey blocks show VFA quantity lost during solids drying. Measured VS removed is 68% higher than actual VS removed.

removed, and Standard Methods (2012) lists VFA-loss as a test interference that underestimates VS. In this study, substantial amounts of VFA were lost when drying samples. Specifically, when the VFA of TS samples were tested before and after drying (following rehydration) two VFA-loss patterns were distinguished (Fig. 3, Panel A). As shown, VFA concentrations expressed in terms of their vapor pressure fraction clustered into the more highly volatile, high concentration (H-Ac, H-Pr, and H-Bu) VFA on one trend line, while the less volatile, low concentration VFA (HiBu and HiVa) were separate; this distinction allowed an empirical fitting (Panel B, Fig. 3) to predict post-drying VFA concentrations based on VFA identity and pre-drying VFA concentration. Least-squares fitted trend lines for pre- and post-drying manure samples indicated that approximately 75% of the VFA moles are lost during drying, although the exact percentage depended on the VFA composition of the sample.

The impact of VFA-loss in the TS/VS analysis is not straightforward. For instance, the difference between measured- and actual-VS removals (Fig. 4) means the productivity expressed as gVFA produced g^{-1}VS removed is underestimated by 40.5%. The opposite occurs in AD0 where the measured VS-removed is underestimated (Fig. 5), and the productivity is overestimated by 12.8%.

Impact of statistical methods

Data distributions in the life sciences are often lognormal rather than normal,[35] so the distribution character of each process metric was tested in this study. However, of all the data sets tested in this study, only the fermenter influent VFA concentrations exhibited a lognormal distribution. If a normal distribution had been assumed, the mean influent VFA would have been 0.236 gVFA day^{-1}, a 15.7% overestimate of the actual 0.204 gVFA day^{-1}.

In addition to data distribution, outliers can introduce significant data interpretation errors; when anomalous processing conditions are identifiable as the cause of outliers, it is generally acceptable to flag and exclude the associated outlier. In this study, outliers were, in fact, detected among the extensive data sets. However, as the contributory processing conditions were not always apparent (that would have allowed a determination on whether to

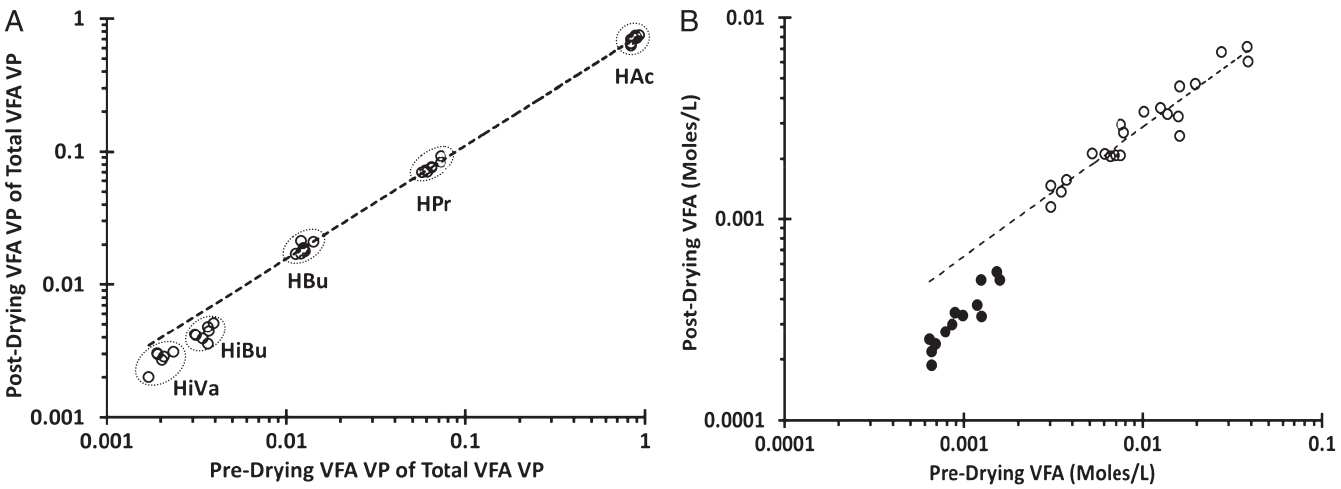

Figure 3. Effect of freezing and thawing on soluble COD (sCOD) released from three different algae batches. Y-axis shows sCOD (g L^{-1}) in algae supernatant, and X-axis is the number of freeze/thaw cycles before sCOD measurement. o = Batch A, □ = Batch B, and Δ = Batch C. Dotted lines run between the means of a pair of measurements. Results standardized on solids concentration of Batch A (17.4%VS).

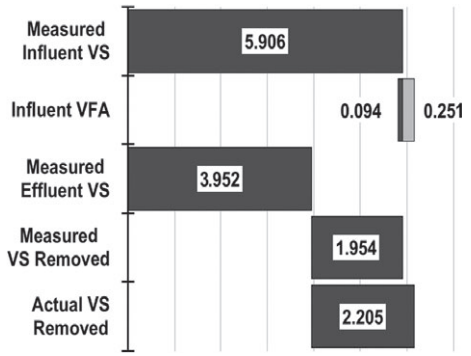

Figure 5. Impact of volatile fatty acids (VFA) loss during volatile solids (VS) testing on the measured performance of the anaerobic digestion of dairy manure. Dark grey blocks show mass of VS or VFA in grams. Light-grey block shows VFA quantity lost during solids drying. Measured VS removed is >11% lower than actual VS removed.

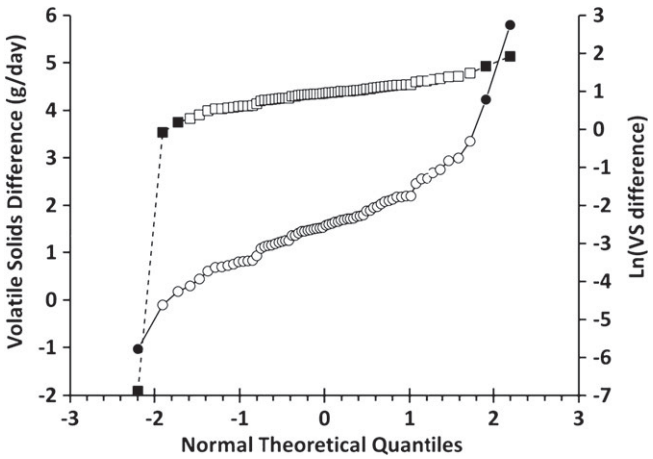

Figure 6. Outlier identification and data distribution of volatile solids (VS) conversion within the experimental fermenter. The Y-axis has VS difference between fermenter effluent and influent VS in g day^{-1}, X-axis has the normal theoretical quantiles, and the second Y-axis has the natural log of the observed VS difference (offset to ensure positive numbers). o = VS difference, □ = ln(VS difference). Filled shapes identified as outliers using Chauvenet's criterion.

include/exclude outliers), this study used Chauvenet's criterion in combination with a visual inspection to flag and eliminate outliers. Chauvenet's criterion has been criticized,[36] but it is simple,[25] objective, and transparent, and has the practical effect of reducing the standard deviation and isolating the more typical performance data (Fig. 6). Visual inspection[24] of each quantile plot generally revealed two distinct data regions; a central portion representing the bulk of the system behavior, and the extremes containing potential outliers. In the example shown (Fig. 6), outliers were easier to detect on the lognormal distribution. Shapiro–Wilk normality testing indicated that the number of data metrics meeting normality criteria was more than five times higher once outliers were removed, and this pattern applied to both normal and log-normal data distributions.

Ranking of potential contributors to literature values

Considering the potential sources of data variability within the context of algal fermentation and AD, it is not possible to say whether these sources played any role in other studies, but an approximate ranking of the phenomena identified and measured would be:

1. There was a 43 to 235% increase in sCOD release from algae after freezing, an impact that could enhance biodegradability.
2. The VFA mass lost during TS/VS testing caused fermenter productivity underestimates of 40%, and AD productivity overestimates of 12.8%.
3. There was a 9 to 28% difference in sCOD amounts released by different algae batches.
4. Fermenter influent VFA concentrations would have been overestimated by 15.6% if a normal rather than lognormal distribution was used.

Ultimately, the authors point out such potential sources of variability to enhance future similar investigations, both by the authors and other investigators.

CONCLUSIONS

The purpose of the research presented and discussed herein was to determine the feasibility and relative productivity of algae grown on PHA-process effluent. Results showed that introducing 10% PHA-algal biomass to fermentation and AD did not cause process disturbances or any statistically significant changes in the methanogenic taxa; algae augmentation produced ~11% more VFA under fermentative conditions and ~11% more methane under AD than dairy manure; and the AD-enriched microorganisms were present in the normal dairy manure biota. Finally, sources of variability need to be controlled in algae conversion tests. For instance, freezing algae can increase sCOD release by more than 200%, and the VFA mass loss during TS/VS measurement affects VS-based metrics.

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