Supplemental Information for

Methane generation and methanogenic community structure in an anaerobic bioreactor for biologically enhanced primary treatment of domestic wastewater under ambient temperatures

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Section 1. Supplemental Methods

Test	Method Used
Total Suspended Solids	Standard Method 2540.D
Volatile Suspended Solids	Standard Method 2540.E
Chemical Oxygen Demand	Standard Method 5220.D using HACH Method 8000 TNT
	822
Biochemical Oxygen Demand	Standard Method 5210.B
Alkalinity	Hach Method 20239 TNTplus 870 (EPA compliant)
Ammonia	Hach Method 10205 HR TNTplus 832 (EPA compliant)
Total Nitrogen	Hach Method 10242 TNTplus s-TKN (EPA compliant)
Nitrate	Hach Method 10206 TNTplus 835 (EPA compliant)
Nitrite	Standard Method 4500-NO ₂ ⁻ using HACH 10237 TNTplus
	840
Dissolved Hydrogen Sulfide	Hach Test Kit HS-WR (223801) Color disc / Methylene blue

1.1. Methods. The list below summarizes methods used during this study.

Note: Nitrate and nitrite results are not presented because each measured concentration was below detectable limits.

1.2. **Dissolved methane measurements**. The method described in Pfluger et al. 2011 was modified for this study. Water samples (approximately 25 mL) were withdrawn from an effluent sampling port located at the top of each reactor compartment directly into a 58-mL serum bottle, which was immediately crimp-sealed with a butyl rubber stopper. The bottle was subsequently shaken to allow methane to equilibrate between the gas phase and the aqueous phase.

1.3. National Center for Biotechnology Information (GenBank)

Project Title: Microbial communities in anaerobic baffled reactor for domestic wastewater treatment

SRA accession SRP136078. Available from: <u>https://www.ncbi.nlm.nih.gov/sra/SRP136078</u>

1.4. Bioinformatic Workflow (available on GITHUB)

Reproducible bioinformatics workflow for project titled "Coupled-Hybrid-Anaerobic-Reactor / Community-Structure-Days-231-395" is available from: <u>https://github.com/Coupled-Hybrid-Anaerobic-Reactor/Community-Structure-Days-231-and-395</u>

Section 2. Supplemental Figures



Figure S1. Schematic of the pilot-scale anaerobic reactor system for treatment of domestic wastewater. The first three compartments in the reactor each have a height-diameter ratio of 12:1, for a total volume of 265 liters. Within each compartment, the hydraulic volume is 240 liters and the headspace is 25 liters. C1, C2, and C3 each have an HRT of 8 hours. The fourth compartment has a height-diameter ratio of 4:1, for a total volume of 89 liters. The hydraulic volume in C4 is 80 liters and the headspace is 9 liters. The total system hydraulic volume for C1 to C4 is 800 liters. The system treats 720 liters of domestic wastewater per day and has total hydraulic retention time of 26.7 hours.



Figure S2. Box and whisker plot for tCOD removal by compartment. The large number of outliers for C1 is a result of highly variable influent concentrations. The number of outliers decreases longitudinally through the reactor. Negative tCOD removal values indicate an increase in tCOD concentrations, which correspond with sampling points when sludge floated to the top of the reactor compartment prior to installation of the gas-liquid-solid separators.



Figure S3. System level sCOD removal and wastewater temperature. (a) Mean monthly sCOD removal with standard deviations graphed against wastewater temperature. sCOD removal increased with when wastewater temperatures and decreased when temperature decreased. (b) Linear regression between weekly mean sCOD removal measurements and wastewater temperature. A weak, but significant, relationship was observed.



Figure S4. Effluent tCOD concentrations (all data points for C3 and C4) compared to the COD-equivalent EPA 30-day secondary discharge standard for this bioreactor system.





Figure S5. Effluent tCOD and wastewater temperature. (a) Mean monthly effluent tCOD concentration (error bars represent one standard deviation) and wastewater temperature over time. (b) Linear regression between weekly mean effluent tCOD measurements and wastewater temperature. A weak, but significant, relationship was observed.



Figure S6. Box and whisker plot for TSS removal by compartment. The large number of outliers for C1 is a result of highly variable influent concentrations. The number of outliers decreases longitudinally through the reactor.



Figure S7. Effluent TSS concentrations (all data points) compared to the EPA 30-day secondary standard. Under warmer temperatures the bioreactor met effluent standards for TSS.





Figure S8. Effluent TSS and wastewater temperature. (a) Mean monthly effluent TSS concentration (error bars represent one standard deviation) and wastewater temperature over time. (b) Linear regression between weekly mean effluent TSS measurements and wastewater temperature. A weak, but significant, relationship was observed.



Figure S9. (A) Observed sludge volume (liters) over time in each compartment of the ABR portion of the ABR-AFFR over time. (B) Sludge volatile solids in each compartment of the ABR portion of the ABR-AFFR over time. A valve failure in C1 on day 241 of the study accounts for the substantial decrease in sludge and volatile solids in C1.

	summer				wir	nter				
Methanosaeta -	0.1	1	4.3	6.5	0.2	5.5	19.6	23.4		
Methanobrevibacter -	1.4	5.4	2.8	2.7	0	1.3	0.9	0.4		
Family Thermoplasmatales Incertae Sedis Genus NA -	0	0.5	1.5	1.4	0	2.2	1.6	2.5	Euryarchaeota	
Methanospirillum -	0	0.1	0.5	1.8	0	1.5	2.3	2.1		
A singuta basedan	10.0	10	0.0	4	20.2	07	4.4	0.2		
	7.0	1.2	0.8	1	29.3	0.7	1.4	0.3		
Acidovolax -	7.8 0.4	3.8	2.1	2.2	8.0	1.7	3.1 0.2	1.7		
Paracoccus -	2.1	6.6	3.3	3.1	0.9	0.3	1.1	0.5		
	5.2	0.0	0.4	1	7.4	0.9	0	0.2	Proteobacteria	
Desulfobulbus -	0	1.2	14	16	0	12	22	2.7		
Desulfovibrio -	11	2	17	1.0	0.7	1.5	0.7	0.9		
Desulfomicrobium -	0	02	0.7	0.3	0	12	1.8	3.1		
		0.2	•	0.0	°,			•		
vadinBC27 wastewater-sludge group -	0	0.8	1.5	2	0	6.3	8.2	13.5		consortium percentage
Macellibacteroides -	0.5	0.8	0.6	1	0	5.6	4.3	4.4	Bacteroidetes	10.0
Cloacibacterium -	4	0.5	0.2	0.4	1.5	0.3	0.3	0.2	Dacterolacies	10.0
Family Prevotellaceae Genus NA -	2.5	0.6	0	0	5	0.3	0.1	0.3		1.0
Romboutsia -	0.9	5.8	7.5	53	0	26	2	16		0 1
Christensenellaceae B-7 group -	0.2	4.3	4.6	42	0 0	5.4	43	21		
Order Clostridiales Family Genus NA -	0	2.4	2.8	24	0	2.8	4.5	1		
Ruminococcaceae NK4A214 group -	0.6	5.5	3.9	4.6	0	0.2	0	0		
Family Acidaminococcaceae Genus NA -	1.6	1.3	0.6	0.6	3.1	1.8	1.6	0.9		
Clostridium sensu stricto 1 -	0.5	2.7	3.4	1.4	1.1	0.6	0.8	0	Firmicutes	
Acidaminococcus -	4.9	1.7	1	1.4	0	0.1	0	0		
Family Veillonellaceae Genus NA -	0.1	1.3	1.9	1.9	0	1.3	1.1	0.6		
- Turicibacter	0.1	2.1	2.8	1.1	0	0.9	0.8	0.5		
Syntrophomonas -	0	0.4	2.3	2.3	0	1.4	0.6	0.5		
			~ -							
Family Synergistaceae Genus NA -	0.1	4.6	8.7	5.7	0	6.6	4.2	3.4		
	0.1	1.9	3.5	3.8	0	9.5	5	3.8	Synergistetes	
	0.3	2.5	2.4	2.2	0	5.4	2.1	1		
Aminomonas -	0	1.1	1.8	2.1			1.3	1.3		
	ent	~	r	ი	ent	~	r	ზ		
×	ll v				INTIN					
				Loc	ation					

Figure S10. Heat map of the most prevalent microorganisms at the genus-level. Organisms are organized within each genus according to greatest net percent relative abundance observed across all locations and times. The tabulated consortium percentage is relative to the entire consortium. The darker red coloration indicates increased relative abundance relative to lighter colors.

Gen

	warm			cold				
Methanosaeta –	3.4	6.9	38.6	53.6	4.4	27.0	76.6	134.8
Methanobrevibacter –	48.0	37.4	25.1	22.2	0	6.4	3.0	2.3
Family Thermoplasmatales Incertae Sedis – Genus Unknown –	0	3.5	13.5	11.5	0	10.8	10.5	14.4
Methanospirillum –	0	6.9	4.5	14.8	0	7.4	9.3	12.1
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Figure S11. Heat map of the absolute abundance of the most prevalent methanogens in the warm- and cold-weather ABR samples (ng DNA g⁻¹ sludge). Absolute abundance of methanogens followed the same trend as relative abundance, with the exception of *Methanobrevibacter* in the influent warm weather sample.

Section 3. Supplemental Tables

		Per	iod 1 (Days 0-1	180)	Period 2 (Days 181-360)			
Variable	Reduction	C1	C2	C3	C1	C2	C3	
1000	Mean	149.3	39.9	15.9	244.7	46.6	66.4	
(mg/L)	95% CI	(53.7, 245.0)	(11.9, 68.0)	(9.5, 22.4)	(135.2, 354.3)	(32.5, 60.7)	(51.7, 81.1)	
(mg/ L)	<i>p</i> -value t-test	0.003	0.011	<0.001	<0.001	<0.001	<0.001	
- 000	Mean	170.6	48.3	0.5	244.6	27.6	39.6	
pCOD	95% CI	(81.9, 259.2)	(25.8, 70.7)	(-11.6, 12.6)	(142.0, 347.2)	(12.8, 42.5)	(25.9, 53.3)	
(mg/ E)	p-value	<0.001	<0.001	0.778	<0.001	0.002	<0.001	
- 000	Mean	-21.2	-8.3	15.5	0.2	19.0	26.8	
sCOD (mg/L)	95% CI	(-4.8, -37.7)	(-28.3, 11.6)	(3.6, 27.3)	(-12.1, 12.4)	(10.0, 27.9)	(20.8, 32.8)	
(mg/ E)	p-value	0.017	0.434	0.012	0.981	<0.001	<0.001	
TOO	Mean	157.9	17.9	3.0	306.1	14.9	19.4	
(mg/L)	95% CI	(58.3, 257.5)	(12.2, 23.6)	(1.3, 4.7)	(138.3, 473.8)	(7.3, 22.5)	(12.5, 26.2)	
(p-value	0.004	<0.001	0.002	0.002	<0.001	<0.001	

Table S1. Mean reduction and 95% confidence intervals (CI) for key performance parameters by compartment of the anaerobic reactor system in different periods of the study. Values in red indicate a lack of statistical significance.

			Period 3 (Da	ays 361-540)			Period 4 (Da	ays 541-720)	
Variable	Reduction	C1	C2	C3	C4	C1	C2	C3	C4
4000	Mean	271.2	47.3	26.3	22.3	170.5	32.9	27.6	16.7
(mg/L)	95% CI	(135.3, 407.1)	(-5.6, 100.2)	(21.1, 31.55)	(18.9, 25.8)	(124.9, 216.1)	(24.7, 41.0)	(21.8, 33.5)	(12.8, 20.6)
(9, =)	<i>p</i> -value	<0.001	0.091	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
= COD	Mean	230.1	47.3	15.0	13.6	112.4	25.1	17.9	9.9
(ma/L)	95% CI	(102.8, 357.4)	(-5.7, 100.2)	(7.8, 22.2)	(8.1, 19.1)	(77.6, 147.2)	(18.0, 32.1)	(11.9, 23.8)	(6.0, 13.8)
(119, 2)	p-value	0.002	0.092	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
-000	Mean	41.1	0.1	11.3	8.7	58.1	7.8	9.8	6.8
(ma/L)	95% CI	(25.0, 57.2)	(-6.7, 6.9)	(4.5, 18.1)	(2.9, 14.5)	(44.0, 72.1)	(3.7, 11.9)	(7.0, 12.5)	(3.8, 9.8)
(119, 2)	p-value	<0.001	0.982	0.003	<0.001	<0.001	<0.001	<0.001	<0.001
тоо	Mean	508.8	18.9	11.5	10.0	170.1	31.9	14.8	6.1
(mg/L)	95% CI	(236.6, 781.0)	(8.6, 781.0)	(8.4, 14.6)	(7.4, 12.5)	(13.0, 327.3)	(25.2, 38.6)	(11.8, 17.7)	(3.8, 8.3)
(9 , =)	<i>p</i> -value	0.001	0.001	<0.001	<0.001	0.044	<0.001	<0.001	<0.001

^a Reduction is defined as the effluent from the previous compartment minus the effluent of the examined compartment (e.g., removal in C1 is the influent concentration minus C1's effluent concentration). A negative value for mean reduction indicates an increase, or generation, of a variable with the reactor compartment.

Table S2. Acetate and sulfate concentrations.

A. Measured acetate and sulfate concentrations in each reactor compartment of the ABR-AFFR. Error bars represent one standard deviation.

Variable	Influent	C1	C2	C3	C4	
Acetate (mg L ⁻¹)	37 ± 21	46 ± 25	44 ± 25	47 ± 24	N/A	
Sulfate (mg L ⁻¹)	59 ± 9	31 ± 9	17 ± 8	12 ± 7	8 ± 6	

B. Statistical significance of sulfate reduction with temperature using t-test (two-sample assuming unequal variances)

Variable	Mean	Variance	Observations	<i>p</i> -value (two-tail)
Temperature (°C)	19.2	11.5	53	1.97 E-40
Sulfate Removal (%)	80	2.6	53	