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Anaerobic digestion of synthetic military food waste-cardboard mixtures in a semi-continuous two-stage system: Electronic Supplementary Information.

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S1. CSTR and UASB COD balances over time

COD flows were monitored over the course of the experimental period. The results are illustrated in Fig. S1. The largest fraction of COD recovery in the CSTR (A) was in the form of large particulate solids in every phase. Small particulate solids comprised less than 1% of COD recovered in Phases I and II, 8-40% in Phase III, 2-20% in Phase IV, and 6-12% in Phase V. Non-VFA sCOD was low in Phases I and II, 3-15% in Phase III, 1-15% in Phase IV, and 3-11% in Phase V. Methane yield was low in Phases I and II, and virtually zero subsequently. VFA yields generally fluctuated between 10-30% in Phases I and II, 5-20% in Phase III, 10-20% in Phase IV, and 4-9% in Phase V. The fluctuations in VFA usually decreased as time progressed in each phase; since no such attenuation occurred in Phase IV and the fluctuations appeared consistent, the representative data for this phase was averaged over a longer sample time period.

The most prominent disruption was when VFA yield spiked to 30-50% of COD recovery between days 105-120, shortly after changing the organic loading rate (OLR) from 8 to 16 g COD L^{-1}. This appears to be a response to the increased loading. Several studies of continuous anaerobic systems have found similar temporary VFA spikes upon introducing new conditions, either with or without a time delay. For example, Gou, et al. [1] observed a spike in VFA at the beginning of their experiment considering codigestion of waste activated sludge and FW that subsided after a few days, even though the initial OLR was low and eventually supported stable methane production. Similarly, Zhang, et al. [2] observed temporary VFA accumulation in the initial days of operation of continuous digesters fed with FW, FW with cattle slurry, and FW with card packaging under a low OLR; in pilot-scale trials, these spikes occurred 20-50 days after the onset of the experimental trial. Fernández, et al. [3] observed VFA accumulation when the OLR of pet food was increased, as expected; however, the spike was delayed by several days

after changing the OLR. Thus, this type of spike appears to be fairly common in anaerobic systems fed with FW-type substrates. It is generally attributed to acidogens adapting more quickly to new conditions than methanogens, resulting in accumulation of fermentation products before the methanogens can begin metabolizing them. The time delays observed in some cases indicate that even the acidogens may require some adaptation time. In the CSTR in this study, the dynamics between the two microbial groups are less important; however, the acidogens likely produced a large amount of VFA once they adapted to the doubled OLR, then became inhibited by the high concentration and resumed a lower rate of conversion.

The UASB varied less between phases (Fig. S1B). Methane generally fluctuated around 100%. The large dips on days 15-25 and 150-160 were attributed to issues with the gas collection apparatus and troubleshooting thereof. The dip between days 105-120 corresponds to the spike in influent COD over the same time period (Fig. S1A). The UASB did not react quickly to the increased loading, but continued producing methane at a lower rate during the spike and resumed near-complete conversion to methane afterwards.

The influent COD (total COD from the CSTR effluent, including VFA, non-VFA sCOD, and small particulate solids) was also plotted on a non-normalized basis alongside methane flow (Fig. S1C). Although the values still show fluctuations and mismatches, it is more apparent that methane production responded to COD inflow. Again, the decreased methane yield is visible in Phase V, where the methane flows fall more consistently below the influent flows.

Overall, these results illustrate that the proposed two-stage reactor system was generally stable, and any temporary disruptions that occurred did not appear to harm the functionality of the consortia. The system would likely be robust to the day-to-day fluctuations expected at a larger scale with real wastes.

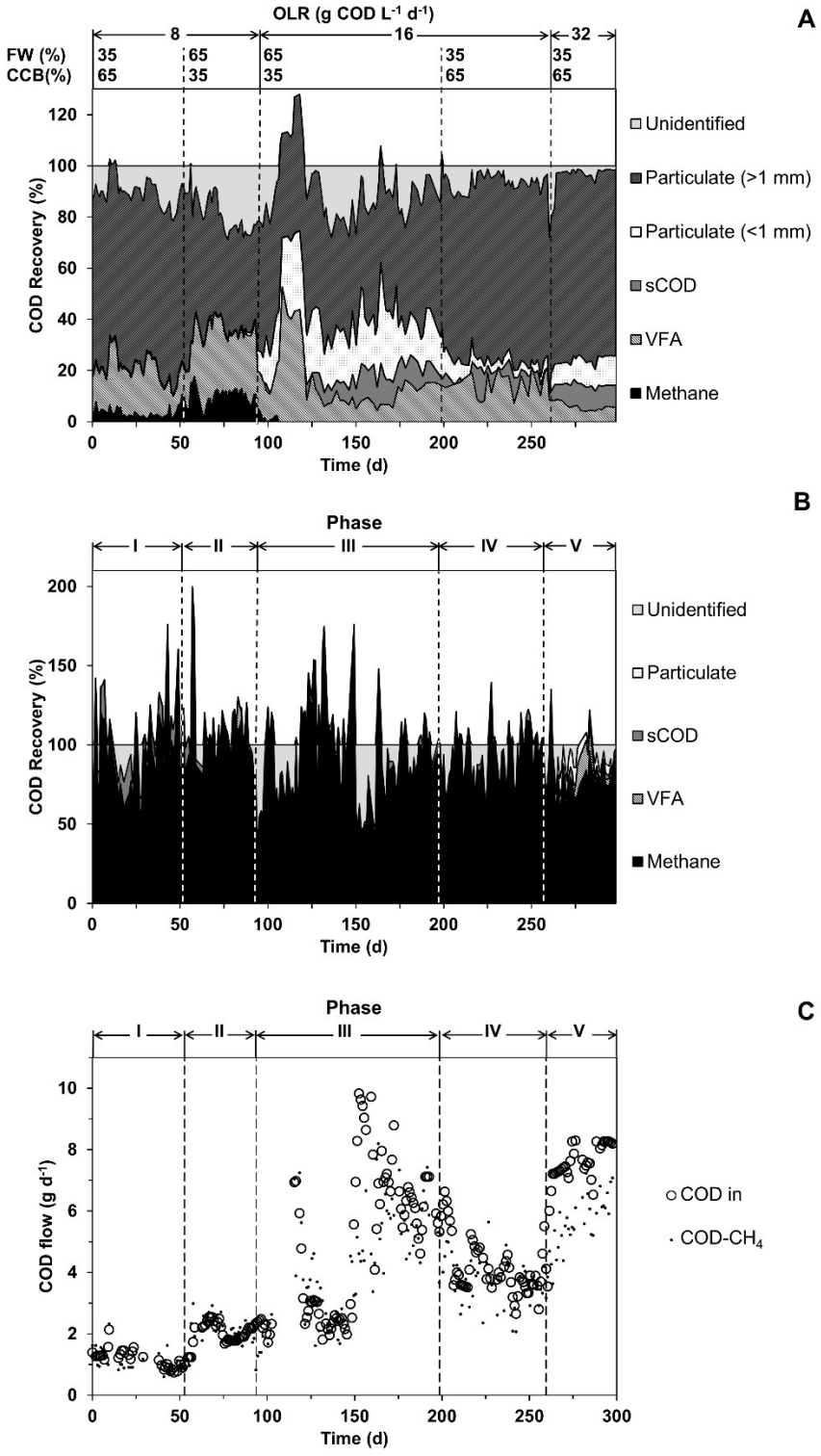

Fig. S1. COD balances normalized by the organic loading rate over the entire experimental period for the CSTR (A) and UASB (B), and non-normalized COD flows in the UASB (C).

S2. Sugar quantification in CSTR effluent

Since non-VFA sCOD comprised larger fractions of COD recovery from the CSTR after Phase III, selected samples from the quasi-steady state periods of Phases III to V were analyzed for sugar content to help determine the nature of this sCOD. Samples from two days in each phase were centrifuged at 13,000 rpm for ten minutes and filtered. High-pressure liquid chromatography (HPLC) analysis was performed using Beckman Coulter System Gold (Brea, CA) equipped with an Aminex HPX-87H column (Bio-Rad; Hercules, CA) and a refractive index detector (JASCO; Easton, MD). Monomeric sugars, cellobiose, and C2-C5 VFAs were quantified.

The sCOD balances by phase are shown in Fig. S2. Sugar concentrations were under 50 mg COD L^{-1} in all phases, so most of the non-VFA sCOD was still unaccounted. Since both FW and CCB are composed mostly of carbohydrates, this suggests that the accumulated non-VFA sCOD was polymeric carbohydrates rather than monomeric sugars. The decreased VFA yields in the last phases were therefore more likely impacted by inhibition of hydrolysis than by inhibition of acidogenesis. This supports the results of ADM1 investigations in which a VFA-dependent inhibition term on hydrolysis improved the model fit to experimental data, while an analogous inhibition term on acidogenesis did not.

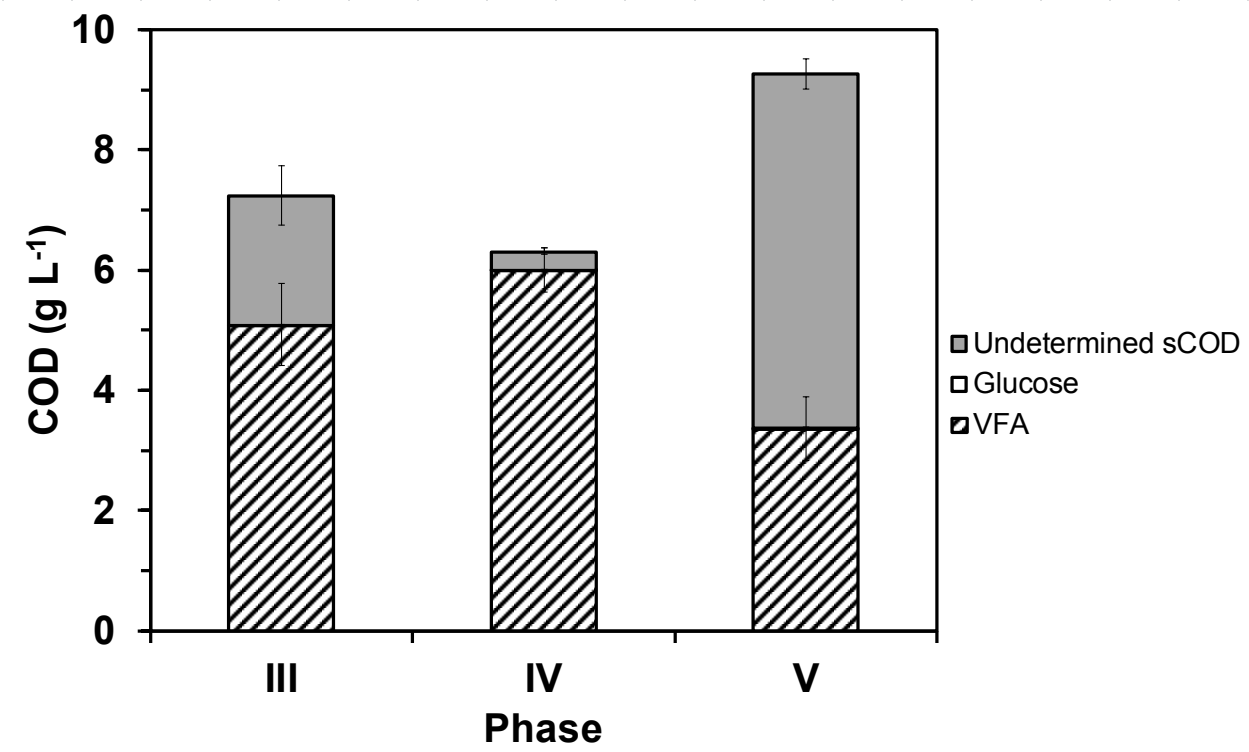

Fig. S2. sCOD balances from quasi-steady state samples of CSTR effluent.

S3. Additional molecular results

The number of sequence reads obtained from each sample are shown in Table S1.

Table S1: Number of sequence reads from molecular analysis of biomass samples

	Bacteria	Archaea
C1	62317	N/A
C2	69850	N/A
C3	68557	N/A
C4	66077	N/A
C5	71151	N/A
U1	79034	77986
U2	73418	129601
U3	58535	136341
U4	49947	85228
U5	58266	75883

Shannon diversity indices, calculated according to Equation S1, are shown in Table S2.

$$H = \sum_i p_i \ln(p_i) \qquad \text{Eq. S1}$$

where H is the Shannon diversity index, i indexes orders comprising at least 1% of at least one sample and p_i is the relative abundance of species i (%).

Table S2: Shannon diversity index (H) for bacterial and archaeal orders

	Bacteria	Archaea
C1	1.25	N/A
C2	1.58	N/A
C3	1.47	N/A
C4	1.21	N/A
C5	1.65	N/A
U1	1.99	0.87
U2	2.20	1.23
U3	2.31	1.03
U4	2.33	1.13
U5	2.25	1.37

Results show that the UASB was consistently more diverse than the CSTR, but there were no obvious trends in H with respect to phase.

S4. Synthetic wastewater recipe

Synthetic wastewater was prepared according to the OECD/OCDE formulation, reproduced in Table S3.

Table S3: Synthetic wastewater recipe [4]

Component	Concentration (mg L^{-1})
Peptone	160
Meat extract	110
Urea	30
K_2HPO_4	28
NaCl	7
$CaCl_2 \cdot 2H_2O$	4
$Mg_2SO_4 \cdot 7H_2O$	2

S5. Sludge characterization methods

Acetoclastic and hydrogenotrophic methanogenic activities were measured by performing batch assays. All assays were performed in an anaerobic medium at an initial pH of 7.2 containing (mg L^{-1}): NH_4Cl (280), $NaHCO_3$ (5000), K_2HPO_4 (250), $CaCl_2 \cdot 2H_2O$ (10), $MgSO_4 \cdot 7H_2O$ (100), $MgCl_2 \cdot 6H_2O$ (90), yeast extract (100) and 1 mL L^{-1} of trace elements. The trace element solution contained (mg L^{-1}): H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (50), $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2000), $NiCl_2 \cdot 6H_2O$ (50), $CuCl_2 \cdot 2H_2O$ (30), $NaSeO_3 \cdot 5H_2O$ (100), EDTA (1000), resazurin (200) and 36% HCl (1 mL L^{-1}).

Assays were performed in 160-mL serum bottles with working volumes of 30 mL and 1 g VSS L^{-1} anaerobic sludge. All bottles were flushed with a gas mix of N_2/CO_2 (80:20, v/v) and then incubated for 3 d at 35 ˚C with mechanical shaking at 120 rpm to allow for endogenous methane production. Next, the bottles were supplied with 1.5 g chemical oxygen demand (COD) L^{-1}) as acetate or H_2. Subsequently, all bottles were flushed and incubated at 35 ˚C at 120 rpm. Gas samples (100 μL) were taken twice or thrice a day to track methane concentration. When cumulative methane production in each assay achieved a plateau for at least two days, experiments were terminated.

Specific acetoclastic or hydrogenotrophic methanogenic activity was taken to be the maximum methane production rate for the corresponding substrate, calculated via linear regression over four consecutive points. These values were corrected with the endogenous methane production rate over the same time interval.

Granule diameter was measured using an Olympus DP72 microscope with cellSens software (Olympus Corporation; Tokyo, Japan). Sludge was strained and immediately examined to avoid loss of moisture content. Over 25 replicate measurements were taken.

To investigate sludge settling, 1 g wet sludge was suspended in water (to avoid clumping) and dropped into a 1000 mL glass graduated cylinder filled with 44 cm of DI water, and the time between dropping the sludge and all the large particles reaching the bottom was recorded. Some fine particles remained in suspension. Six replicates were performed. The minimum sedimentation velocity was taken to be the height of the water column divided by the time for all coarse particles to settle.

References

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3. Fernández, A.; Sánchez, A.; Font, X., Anaerobic co-digestion of a simulated organic fraction of municipal solid wastes and fats of animal and vegetable origin. *Biochem. Eng. J.* **2005,** *26*, (1), 22-28.
4. OECD/OCDE, OECD Guideline for the Testing of Chemicals. In *Simulation Test - Aerobic Sewage Treatment*, Organization for Economic Co-operation and Development: Paris, France, 2001; pp 1-50.