Supplementary Materials

Description

This document supports the information described in the main text by detailing:

1. The methods used in reactor monitoring (Table S1);
2. The calculation protocol used to obtain the specific organic loading rate (sOLR);
3. The procedures used in molecular analyses;
4. The heat maps showing the relevant genera in biomass samples collected from the thermophilic methanogenic reactor (RMT) (Figure S1) and from the mesophilic methanogenic reactor (RMM) (Figure S2).

S1. Analytical Methods Used in Reactor Monitoring

The analytical methods used in reactor monitoring are listed in Table S1.

Table S1. Analytical methods were used in the monitoring of the methanogenic reactors (liquid, gas, and solid phases included).

Parameter	Method	Reference
Chemical oxygen demand (COD)	Colorimetry	APHA et al. [19]
Sulfate (SO_4^{2-})	Turbidimetry	APHA et al. [19]
Volatile organic acids (VOA)	Titration	Kapp [84]
Total phenols (PheOH)	Colorimetry	Buchanan and Nicell [85]
Total carbohydrates	Colorimetry	Dubois et al. [86]
Lactic acid	Colorimetry	Taylor [87]
Volatile organic acids [1] and solvents [2]	Gas chromatography	Adorno et al. [88]
Volatile suspended solids (VSS)	Gravimetry	APHA et al. [19]
Biogas composition (CO_2, CH_4, H_2S, N_2)	Gas chromatography	Lebrero et al. [89]

[1] Acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, and caproic acids.

[2] Acetone, methanol, ethanol, and butanol.

In addition, the microscopy image of the granular-like aggregates collected from RMM was obtained in a scanning electron microscope model 440 (LEO Electron Microscopy Ltd., Oxford, UK), which was also used in the energy-dispersive X-ray spectroscopy analysis.

S2. Specific Organic Loading Rate Calculation

The specific organic loading rate (sOLR), which represents the temporal dynamics of the food-to-microorganism (F/M) ratio (or the biological load) in continuous systems, was calculated using the methodology initially described by Anzola-Rojas et al. [20] and further modified elsewhere [21]. The biomass growth yield coefficient ($Y_{X/S}$; g VSS g^{-1}COD) was initially calculated using Eq. (S1), in which the terms Xr_f, Xr_0, X_e, and mCOD correspond, respectively, to the amount of biomass retained in the reactors (FDZ + STB) at the end of the operating period (g VSS), the amount of biomass in the reactors after the inoculation period, i.e., at time zero (g VSS), the amount of biomass washed out during the entire continuous operating period (days 0–171) (g VSS), and the total amount of converted substrate during the entire continuous operating period (days 0–171) (g COD). COD refers to the total chemical oxygen demand assessed in non-centrifuged samples. The terms X_e and mCOD were calculated using Eqs. (S2) and (S3), respectively, in which the terms VSS_e, Q, COD_{inf}, and COD_{eff} are, respectively, the effluent VSS concentration (g L^{-1}), the liquid phase flow rate (L d^{-1}), and the COD in the influent and in the effluent streams (g L^{-1}).

$$Y_{X/S} = \frac{Xr_f - Xr_0 + Xe}{mCOD} \tag{S1}$$

$$Xe = \int_0^{171} (VSSe \times Q)dt \tag{S2}$$

$$mCOD = \int_0^{171} \left[\left(COD_{inf} - COD_{eff} \right) \times Q \right]dt \tag{S3}$$

The biomass concentration in the reactors at a given time "n" (X_n, g VSS L^{-1}) was calculated using Eq. (S4), in which the terms Δt, fXr, V_w, and X_{n-1} are, respectively, the period of time (d) between two consecutive measurements of VSS_e, COD_{inf}, and COD_{eff}, the fraction of retained biomass (dimensionless), the working volume of the reactors (L), and the biomass concentration in the reactors at a time "n − 1." fXr represents the ratio between the amount of biomass produced and retained in the reactors during the operation and the total amount of biomass produced during the operation (Eq. (S5)). Using this factor implies considering that a fixed proportion of the produced biomass was retained in the reactor throughout the operation. The biomass retention rate (BRR, g VSS L^{-1} d^{-1}) was calculated by differentiating the temporal profiles of X_n. Finally, the sOLR at a given time "n" ($sOLR_n$, g COD g^{-1}VSS d^{-1}) was calculated by Eq. (S6), in which the term OLR is the applied organic loading rate (g COD L^{-1} d^{-1}) in the period.

$$X_n = \left[\frac{\left(COD_{inf} - COD_{eff} \right) \times Q \times Y_{X/S} \times \Delta t}{V_W} \right] \times fXr + X_{n-1} \tag{S4}$$

$$fXr = \frac{Xr_f - Xr_0}{Xr_f - Xr_0 + Xe} \tag{S5}$$

$$sOLR_n = \frac{OLR}{X_n} \tag{S6}$$

The sOLR was also assessed separately in the FDZ (sOLR$_{FDZ}$) and in the STB (sOLR$_{STB}$) of the reactors according to Eq. (S7) (sOLR$_{FDZ,n}$) and Eq. (S8) (sOLR$_{STB,n}$), respectively. In Eq. (S7), the term $Xr_{f,FDZ}/Xr_f$ corresponds to the ratio between the amount of biomass retained in the FDZ ($Xr_{f,FDZ}$) and the total amount of biomass (FDZ + STB) retained in the

reactor, both measured at the end of the operation during reactor disassembly. In turn, the term $Xr_{f,STB}/Xr_f$ in Eq. (S8) is the ratio between the amount of biomass in the STB and the total amount of biomass (FDZ + STB), while $ER_{COD,FDZ}$ is the COD removal efficiency in the FDZ (values listed in Table 1 of the main text). All calculations involving the integration or differentiation of curves were carried out using the software Origin 2020 (OriginLab Corporation, Northampton, MA, USA).
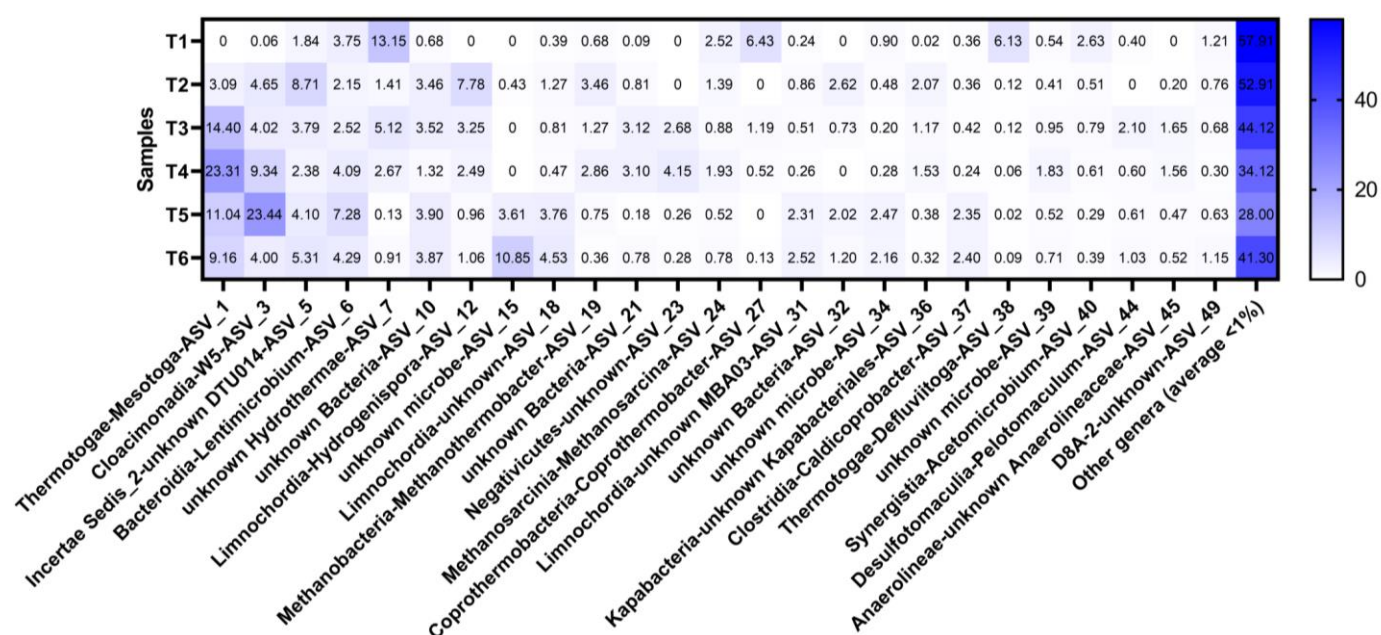
$$sOLR_{FDZ,n} = \frac{COD_{inf} \times Q}{\left(\frac{Xr_{f,FDZ}}{Xr_f}\right) \times X_n \times V_w} \tag{S7}$$

$$sOLR_{STB,n} = \frac{\left(1 - ER_{COD,FDZ}\right) \times COD_{inf} \times Q}{\left(\frac{Xr_{f,STB}}{Xr_f}\right) \times X_n \times V_w} \tag{S8}$$
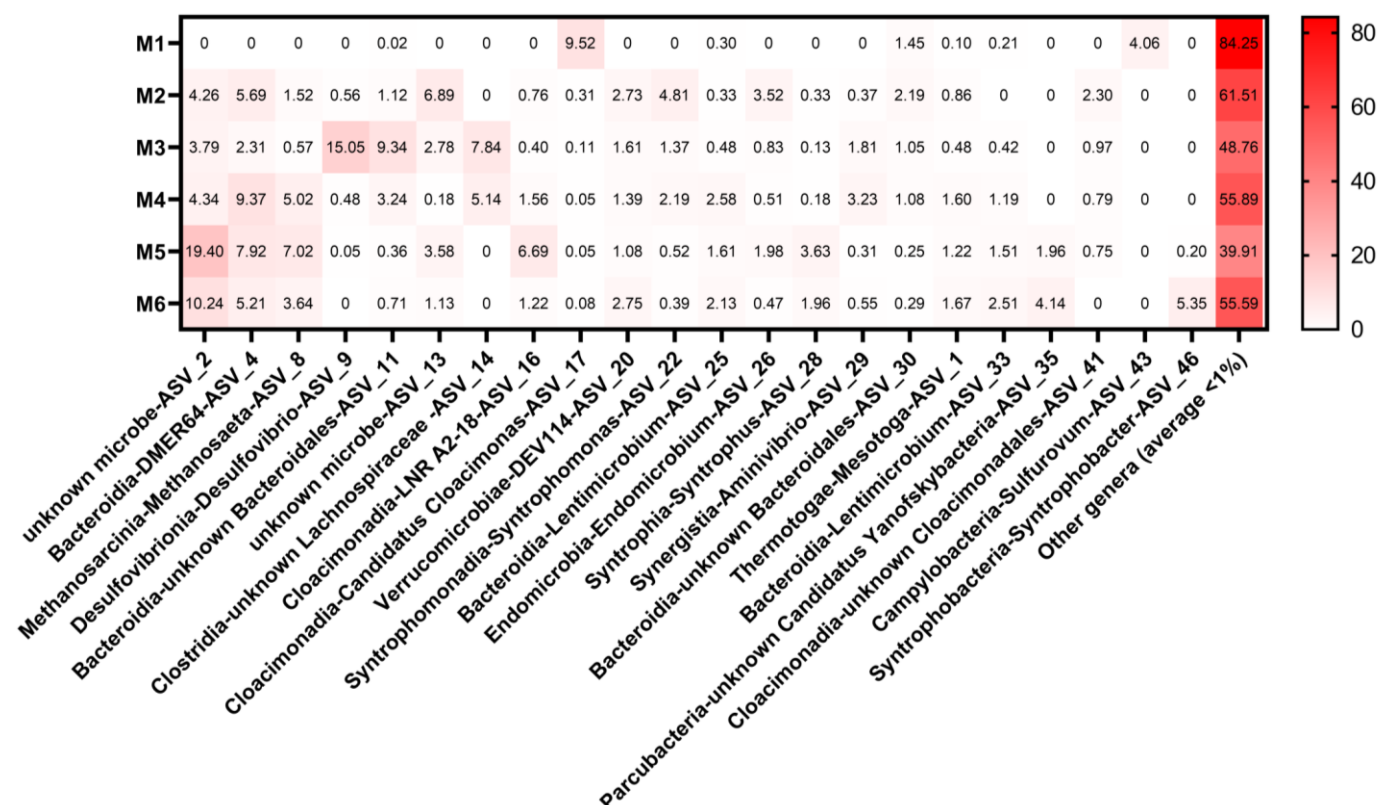
S3. DNA Extraction, 16S rRNA Gene Amplicon Sequencing and Bioinformatics (According to Piffer et al. [98] and Fuess et al. [1])

The FastDNA SPIN Kit for soil (MP Biomedicals, Irvine, CA, USA) was used in DNA extraction, following the manufacturer's instructions. Approximately 0.5 g of wet biomass from each sample was used. Genomic DNA was analyzed on both quantitative and qualitative bases in a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) using gel electrophoresis on 1.8% agarose. The extracted genomic DNA was stored at −20°C prior to sequencing. The universal primers 515F and 806R targeting the V4 region of the 16S rRNA gene for bacteria and archaea [90] were used in the polymerase chain reaction (PCR). Libraries were prepared using 2 µL of stock DNA, 0.2 nM of each primer, 1x of 2x PCRBio Ultra Mix (PCR Biosystems Inc., Wayne, PA, USA), and ultrapure water to complete a final volume of 25 µL. Reactions were performed in a thermocycler with an initial 3 min denaturation step at 94 °C, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and a final elongation at 72 °C for 10 min. The holding temperature was maintained at 4 °C. PCR products were purified with AMPure XP Beads (Beckman Coulter Inc., Brea, CA, USA). The 16S rRNA gene amplicon libraries were further sequenced using a MiSeq™ System (Illumina, Inc., San Diego, CA) owned by NGS Soluções Genômicas (Piracicaba, SP, Brazil).

Sequence analysis was performed in R [91] and Linux bash. Primer sequences that are known to interfere in amplicon sequence variant (ASV) detection were excluded using Biostrings [92] and Cutadapt [93]. Sequences with Ns were removed in DADA2 [94]. Each individual file was dynamically trimmed with SolexaQA++ [95]. DADA2 was also used in R to predict errors, calculate ASV, and merge pairs. Finally, merged sequences were annotated using the DECIPHER package [96] against the SILVA 16S rRNA genes (version 138.1) [97], with calculations performed in R.

Figure S1. Heat map analysis of the most abundant genera in biomass samples collected from the thermophilic inoculum (T1) and from RMT (T2–T6). Please refer to Table 3 (main text) for details on the biomass sample description.

Figure S2. Heat map analysis of the most abundant genera in biomass samples collected from the mesophilic inoculum (M1) and from RMM (M2–M6). Please refer to Table 3 (main text) for details on the biomass sample description.