



# Enhanced acidogenic fermentation of waste sludge via hydrothermal carbonization pretreatment: Process optimization, performance limitations, and carbon pathways

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## ABSTRACT

Waste sludge management poses major economic and environmental challenges. Valorization through acidogenic fermentation to generate volatile fatty acids (VFAs) offers a sustainable alternative, but the low biodegradability of waste sludge often requires pretreatment to enhance process efficiency. This study investigated hydrothermal carbonization (HTC) as a pretreatment to improve VFA production from waste sludge via subsequent thermophilic fermentation (50 °C). Biochemical methane potential tests were used to optimize HTC conditions (170–200 °C, 0–120 min), identifying 180 °C for 60 min as the most effective for enhancing sludge biodegradability. Continuous fermentation of HTC-pretreated waste sludge (applied either as whole slurry or as separated process liquid) was then carried out in completely stirred tank and sequencing batch reactors. HTC pretreatment increased VFA production and enriched the mixture in longer-chain acids (butyric, valeric, caproic; 30–40 % of total VFAs), which are advantageous for downstream applications such as polyhydroxyalkanoate synthesis. Nonetheless, moderate acidification yields (~0.3 gCOD-VFAout/gCODs,out) indicated incomplete conversion, likely due to recalcitrant organics in the HTC-treated sludge, as confirmed by GC-MS analyses. Both process configuration and hydraulic retention time markedly influenced performance and microbial community composition. COD mass balance revealed substantial carbon partitioning into the hydrochar fraction, underscoring the importance of its valorization to ensure overall process sustainability. In summary, HTC shows potential as a pretreatment strategy for converting waste sludge into VFAs, but addressing the limitations imposed by recalcitrant compounds is crucial to maximize carbon recovery within a circular economy framework.

## 1. Introduction

Wastewater treatment plants (WWTPs) generate substantial amounts of waste sludge, posing significant management challenges due to high treatment and disposal costs, accounting for up to 50 % of total WWTP operational costs [1]. Despite its complex composition including organic matter, microorganisms, water, and solids, waste sludge represents a renewable resource with potential for energy and material recovery [2]. As global waste sludge production continues to rise, driven by stricter environmental regulations and expanding wastewater infrastructure, particularly in developing countries [3], the development of efficient and sustainable valorization strategies for resource recovery is crucial

for both economic and environmental sustainability, particularly in light of net-zero targets in wastewater treatment [1,4].

Common valorization approaches include biogas and biofuel production, nutrient recovery, and the generation of bio-based materials such as bio-based fertilizers and sorbents [5]. Among these, anaerobic fermentation for volatile fatty acid (VFA) production has gained significant interest in recent years. VFAs are valuable resources to recover as they serve as versatile platform chemicals with applications in biological nutrient removal and biopolymer synthesis, particularly for polyhydroxyalkanoates, a promising biodegradable alternative to fossil-based plastics [6].

To enhance waste sludge anaerobic fermentability, a variety of

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pretreatment methods, including physical, chemical, and thermal approaches, have been explored to accelerate hydrolysis, the rate-limiting step in anaerobic bioconversion, thereby increasing the bioavailability of organic matter for microbial conversion [7]. Hydrothermal carbonization (HTC) has emerged as a promising thermochemical pretreatment technology that can improve waste sludge dewaterability and subsequent biochemical conversion potential [8]. Typically conducted at elevated temperatures (170–350 °C) and autogenous pressures (5–25 MPa), HTC involves a series of complex chemical reactions like hydrolysis, dehydration, decarboxylation, and depolymerization. This process yields two main products: hydrochar, a carbon-rich solid with potential applications as a biofuel or soil amendment, and a process liquid containing dissolved organic compounds [9,10]. It is critical to note that while the term "carbonization" implies the formation of a stable, inert solid, at the milder temperatures (<200 °C) relevant for enhancing biological processes, the primary effect is an aggressive thermal hydrolysis that solubilizes and liquefies the organic matter rather than extensively charring it.

Although hydrochar production has been the primary focus of many HTC studies [8], the process liquid retains a significant fraction (20–50 %) of the initial organic matter, often enriched with soluble and readily biodegradable compounds such as sugars, amino acids, and VFAs [11,12]. While frequently considered a byproduct requiring further treatment, this liquid phase represents a valuable substrate for subsequent fermentation processes, potentially enhancing overall carbon recovery and maximizing VFA yields [9]. Recent studies suggest that specific HTC conditions, particularly lower temperatures (e.g., 170 °C), can favour the solubilization of carbohydrates and proteins, thereby potentially boosting fermentation performance [9]. However, research specifically investigating the fermentability of HTC-treated waste sludge remains limited to date, and existing studies have reported conflicting results regarding VFA production efficiency [8,13].

While previous studies have investigated HTC and acidogenic fermentation separately, a comprehensive and integrated evaluation of this combined process pathway is still lacking. Key questions remain regarding the optimal pre-treatment conditions for fermentation, the performance limitations in continuous operation, and the overall carbon balance of a system that produces two distinct product streams. Therefore, the primary objectives of this study were to:

- (1) Identify the optimal HTC conditions (temperature and time), starting from prior research indicating potential benefits for solubilization [9,14], not for solid fuel production, but specifically to maximize the anaerobic biodegradability of waste sludge for subsequent biological valorization.
- (2) Conduct a comprehensive, multi-faceted investigation of the continuous acidogenic fermentation of the optimally pre-treated waste sludge, deconstructing the influence of reactor configuration (SBR vs. CSTR), feedstock type (whole slurry vs. separated process liquid), and hydraulic retention time on VFA production and its limitations.
- (3) Perform a complete COD mass balance of the entire integrated process to quantify the partitioning of carbon between the liquid (VFAs, residual organics), solid (hydrochar), and gaseous (methane) phases, thereby providing critical insights for full-scale implementation and a comprehensive biorefinery concept.

By addressing these objectives, this research seeks to clarify the potential and challenges of this combined approach for sustainable waste sludge management and resource valorization, contributing to the advancement of circular economy principles within the water sector.

## 2. Materials and methods

### 2.1. Waste sludge and microbial inoculum source

The feedstock used throughout this study for the HTC was waste activated (secondary) sludge, hereafter referred to simply as waste sludge. The waste sludge for HTC and the microbial inoculum for acidogenic fermentation were collected from a WWTP located in northern Italy. The WWTP serves a population equivalent of fewer than 10,000 and treats municipal wastewater using a conventional process line comprising primary settling, biological treatment (predenitrification followed by aerobic oxidation/nitrification), and secondary settling. Waste sludge was collected from an equalization tank prior to thickening and it was then concentrated to approximately 5 % total solids by via screw press to enhance its organic content. The microbial inoculum used in the fermentation experiments was obtained from the WWTP's anaerobic digester, which treats both primary and secondary sludge.

### 2.2. Sludge pretreatment

The thickened waste sludge underwent HTC in a custom-built, 5-L maximum capacity, high-pressure lab-scale reactor (B-Plas). The reactor was equipped with an electric heater regulated by a Proportional-Integral-Derivative controller using feedback from a PT100 temperature sensor. An internal cooling coil circulating water was used to control temperature ramps and facilitate post-treatment cooling. Investigated HTC conditions (temperature, holding time) are detailed in Sections 2.3, with the holding time referring to the duration for which the HTC reactor maintains the target temperature after the initial heating phase.

Following the HTC treatment, the resulting HTC slurry was subjected to pressure filtration to separate the process liquid fraction (HTC PL) from the solid hydrochar residue and to assess dewaterability. A bench-scale pressure filter apparatus (filtration area of 0.0077 m<sup>2</sup> per side) was used to simulate single-cake filtration conditions. Compressed air, regulated by a pneumatic panel, was used to exert pressure. A filter cloth with permeability ≤ 50 L/m was employed. The filtration procedure involved feeding the HTC slurry for 1 h, followed by a 15-minute cake-squeezing step at 10 bars. This process aimed to achieve a final cake with a target moisture content of approximately 50 %.

### 2.3. BMP tests

BMP tests were conducted to evaluate the effect of the HTC pretreatment on the ultimate methane yield of the waste sludge, serving as an indicator of overall anaerobic biodegradability changes. Tests were performed on untreated waste sludge, the HTC slurry obtained after the HTC treatment, the hydrochar and the HTC PL fractions obtained after separation of the HTC slurry. Tests were run under mesophilic conditions (35 °C) using an automated volumetric measurement system (AMPTS II, Bioprocess Control), generally following the UNI/TS 11703:2018 standard. To ensure reproducibility, all BMP assays were conducted in duplicate.

Three sets of experiments were conducted:

- (i) To assess the effect of HTC temperature (170, 180 and 200 °C applied for a holding time of 60 min) on the biodegradability of HTC slurry.
- (ii) To assess the effect of HTC holding time (180 °C applied for a holding time of 0, 30, 60 and 120 min) on the biodegradability of HTC slurry.
- (iii) To assess the biodegradability of HTC products (HTC slurry, HTC PL and hydrochar) treated at 180 °C for a holding time of 60 min compared to the untreated waste sludge.

## 2.4. Acidogenic fermentation tests

Acidogenic fermentation was carried out in four process configurations operated in continuous mode under thermophilic conditions (50 °C), namely one SBR and three CSTRs. Each configuration was operated as a single, long-term experiment. Performance was evaluated after a stable, pseudo-steady state was achieved (typically after an operational period exceeding 5–10 times the SRT), with this long-term stability serving as the basis for demonstrating the process's replicability under the tested conditions. The SBR was operated with three identical 8-hour cycles per day. Each cycle consisted of sequential phases: influent feeding (1 min), anaerobic reaction under complete mixing (417 min), sludge purge (1 min), biomass settling (60 min), and effluent discharge (1 min). A portion of sludge was withdrawn during the mixing phase to maintain the SRT at the target value. The three CSTRs were operated with an intermittent flow regime, involving feeding and simultaneous withdrawal of an equal volume of mixed liquor, so that the SRT equated the HRT. For temperature control ( $\pm 1$  °C), CSTR1 and CSTR2 were put on a heating pad connected to a temperature controller while SBR and CSTR3 were immersed in a thermos-regulated bath. All reactors were stirred magnetically, and gas collection was achieved using a Tedlar bag. A summary of the experimental design for investigated configurations, including running time, reactor volumes, HRT and sludge retention time (SRT) values, organic loading rates (OLR) expressed in terms of soluble chemical oxygen demand (sCOD) and volatile solids (VS), and feed type, is presented in Table 1. Throughout the experiment, the reactors were fed sequentially with feedstock prepared through HTC treatment of raw waste sludge sources collected in different days, resulting in four distinct batches for SBR, CSTR1, and CSTR2, and three batches for CSTR3. Since the CSTRs were operated in parallel, the feedstock batches used for these three reactors were synchronized. To account for potential batch variability, each batch of feedstock (both HTC slurry and HTC PL) was characterized prior to use. Key parameters including tCOD, sCOD, ammonium (NH<sub>4</sub><sup>+</sup>), phosphate (PO<sub>4</sub><sup>3-</sup>), VFAs, total solids (TS), VS, total suspended solids (TSS), and volatile suspended solids (VSS) were determined for each batch to ensure process monitoring was based on the specific characteristics of the influent.

Furthermore, the microbial inoculum used in all the reactors was heat-treated at 80 °C for 1 h to suppress methanogenic activity and promote the enrichment in acidogenic bacteria. Subsequently, during the initial acclimation phase (approximately 15 d) of the acidogenic fermentation reactors, 2-bromoethanesulfonic (BES) acid, a methanogen inhibitor, was fed at a concentration of 0.2 g/L.

Two fermentate samples were collected weekly from each reactor and characterized for pH, sCOD, NH<sub>4</sub><sup>+</sup>, PO<sub>4</sub><sup>3-</sup>, VFAs, TS, VS, TSS, and VSS. Biogas production volume was determined by regularly measuring the volume of the collection Tedlar bags. Separately, the biogas composition (CH<sub>4</sub>, CO<sub>2</sub>, H<sub>2</sub>S) was analyzed on a weekly basis.

## 2.5. Analytical methods

### 2.5.1. Chemical-physical characterization

TS, VS, TSS, and VSS were determined in duplicate according to Standard Methods 2540 G, 2540 E, and 2540 D, respectively [15]. pH was measured using a portable meter and probe (HACH HQ40d). For sCOD, N-NH<sub>4</sub><sup>+</sup>, and P-PO<sub>4</sub><sup>3-</sup> analyses, samples were first centrifuged (4,

000 × g, 10 min) and the supernatant filtered through 0.45 μm pore size filters. The filtered samples were then analyzed using commercial colorimetric test kits (HACH LCK014 for sCOD, LCK302 for NH<sub>4</sub><sup>+</sup>, LCK350 for PO<sub>4</sub><sup>3-</sup>). Total COD (tCOD) of HTC slurry and HTC PL was determined using the potassium dichromate closed reflux colorimetric method (APAT/IRSA-CNR 5130 protocol).

Individual VFAs (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, iso-caproic, caproic acids) and ethanol concentrations were determined according to Standard Methods 5560 [15] using gas chromatography (GC). The GC system (DANI Master GC) was equipped with a flame ionization detector (FID 86/10). Biogas composition (CH<sub>4</sub>, CO<sub>2</sub>, H<sub>2</sub>S) was analyzed using a separate GC system (DANI Master GC) equipped with a thermal conductivity detector (TCD 86/40).

### 2.5.2. Data processing

Process performance for acidogenic fermentation tests was evaluated using the following metrics. The acidification yield (Y<sub>A</sub>) was calculated as the ratio between the net production of VFAs and the total soluble COD in the influent, as reported in Eq. 1.

$$Y_A = \frac{gVFA_{OUT} - gVFA_{IN}}{gCOD_{s,IN}} * 100 \quad (1)$$

Where gVFA<sub>OUT</sub> and gVFA<sub>IN</sub> are the mass of VFAs expressed as COD of the effluent and the influent, respectively, while gCOD<sub>s,IN</sub> is the mass of COD of the influent to the acidogenic fermentation reactor. Acidification efficiency (E<sub>A</sub>) was calculated as the ratio between the mass of VFAs (gVFA<sub>OUT</sub>) and the mass of soluble COD (gCOD<sub>s,OUT</sub>) in the effluent, according to Eq. 2.

$$E_A = \frac{gVFA_{OUT}}{gCOD_{s,OUT}} \quad (2)$$

The VFA volumetric productivity (P) was calculated as the ratio between the net production of VFAs and the HRT.

$$P = \frac{VFA_{OUT} - VFA_{IN}}{HRT} \quad (3)$$

Where VFA<sub>OUT</sub> and VFA<sub>IN</sub> are the concentrations of VFAs expressed as gCOD/L of the effluent and influent, respectively.

To determine if observed differences in key fermentation parameters were statistically significant, *t*-tests were performed using a significance level (α) of 0.05 using Microsoft Excel.

### 2.5.3. Process mass balance

To evaluate the overall efficiency of integrating HTC pretreatment with acidogenic fermentation, a COD mass balance was constructed for each process configuration. All COD flows were normalized relative to the initial total COD of the untreated waste sludge fed to the HTC process (COD<sub>0</sub>). The mass balance accounted for three main COD fractions: directly measured sCOD, a portion of which was specifically quantified as sCOD-VFA; particulate COD (pCOD); and the COD equivalent of methane. Particulate COD was calculated as the difference between tCOD and sCOD for any stream where both were measured, while the methane COD was calculated from its production volume using the theoretical conversion factor (e.g., 2.86 g COD/L CH<sub>4</sub>, [16]). The COD partitioning within the HTC plus pressure filtration stage was

**Table 1**

Experimental design of acidogenic fermentation tests for investigated configurations: running time, reactor volume, HRT, SRT, OLR and type of feed.

	Running time	Reactor volume	HRT	SRT	OLR (avg. ± st. dev.)		Feed type
	[d]	[L]	[d]	[d]	[g sCOD <sub>IN</sub> L <sup>-1</sup> d <sup>-1</sup> ]	[g VS <sub>IN</sub> L <sup>-1</sup> d <sup>-1</sup> ]	-
SBR	108	0.7	2	3.2	11.2 ± 1.3	19.7 ± 5.4	HTC PL
CSTR1	84	2	4	4	6.7 ± 1.2	7.5 ± 1.0	HTC slurry
CSTR2	84	2	4	4	3.5 ± 0.5	4.6 ± 0.3	HTC PL
CSTR3	52	0.7	8	8	2.4 ± 0.02	3.6 ± 0.2	HTC slurry

determined by a differential approach. Specifically, the COD of the solid hydrochar was calculated indirectly by subtracting the total mass of COD measured in the HTC PL the total mass of COD measured in the HTC slurry. This method was preferred over the direct measurement of the solid hydrochar cake due to the inherent difficulty in obtaining a perfectly homogeneous and representative subsample from a dewatered solid.

#### 2.5.4. Preliminary techno-economic analysis

A preliminary techno-economic analysis was conducted to compare the annual operational expenditure (OPEX) of the proposed integrated HTC process against a conventional sludge management line. The analysis was based on a full-scale case study processing 40,000 tons/year of municipal waste activated sludge, with process conditions mirroring those investigated in this study (HTC at 180 °C for 60 min followed by filter press dewatering). The conventional line was defined as sludge dewatering via centrifuge.

The OPEX for both scenarios was calculated by summing the annual costs of six key components: cake transport and disposal, maintenance, operator labor, polyelectrolyte consumption, electrical energy, and thermal energy. Cost data were derived from a combination of sources to reflect real-world conditions for a plant located in Italy: costs for cake management, maintenance, and operator labor were based on actual client data, while unit costs for energy were based on recent published European market data (Eurostat for natural gas; market analysis for electricity). The analysis considered the economic impact both within the plant gate and for the total cost including off-site transport and final disposal. The payback period was estimated by considering the operational savings against typical capital investment for a plant of this scale.

#### 2.5.5. Microbiological characterization

Total genomic DNA was extracted from acidogenic fermenter biomass samples using the DNeasy 96 PowerSoil Pro Kit (Qiagen) on a Qiacube HT automated platform. For each extraction, approximately 50–100 mg of sludge sample, 100 µL of garnet beads (provided with the kit), and 800 µL of the kit's lysis solution (Solution CD1) were added to the sample tubes. Samples were incubated at 76 °C for 15 min, followed by mechanical lysis in a TissueLyser II instrument (Qiagen) at 25 Hz for 10 min. After lysis, samples were centrifuged at 16,000 × g for 1 min. 550 µL of the resulting supernatant was transferred to the Qiacube HT for automated DNA purification according to the manufacturer's protocol.

5 µL of DNA was used for amplification with the Platinum HiFi taq (ThermoFisher) using the following amplification protocol: 94 °C for 2 min, followed by 25 cycles at 94 °C for 30 s, 55 °C for 30 s and 68 °C for 30 s, and a final extension at 68 °C for 7 min). The modified primers were Pro341F and Pro805R, from [17]. The amplicons were tested in 1.5 % agarose gels to verify the concentration. The amplicons were purified with Thermolabile Esonuclease I (NEB), diluted 1:2 and amplified with short cycle with Nextera XT Indexes. The amplicons were then normalised with SequalPrep (Thermo Fisher) and multiplexed into pools, concentrated and purified with Agencourt XP 1X Magnetic Beads and verified with Qubit and Agilent Bioanalyzer HS. The pooled library was sequenced on an Illumina MiSeq platform using a MiSeq Reagent Kit v3 with an approximate 20 % PhiX control library spike-in.

#### 2.5.6. Chemical speciation of substrates and reaction intermediates

To characterise the organics delivered by HTC and to assess the actual biodegradability of each constituent, detailed GC-MS analyses (with and without trimethylsilylation) were performed onto HTC PL and effluent from CSTR3 as detailed elsewhere [18]. Most relevant compounds (e.g. cyclic dipeptides of pyrazined) were manually integrated and quantified using internal calibration based on commercial standards, whereas chemical classes of compounds were quantified as groups, using automated peak grouping and resolution as detailed in [19].

### 3. Results and discussion

#### 3.1. BMP tests

Besides evaluating the biodegradability and biomethane production of investigated substrates (untreated waste sludge, HTC slurry, HTC PL and hydrochar) under anaerobic digestion conditions, the objective of BMP tests was to optimize the temperature and holding time of the HTC treatment to enhance waste sludge biodegradability. To identify the optimal HTC temperature, a set of BMP tests was performed to compare untreated waste sludge and HTC slurry pretreated at 170, 180 and 200 °C (all for a 60-minute holding time). Experimental results, presented in Fig. 1a, confirmed that HTC enhanced anaerobic biodegradability across the tested temperature range, with 180 °C yielding the highest BMP value of 243.2 NmL CH<sub>4</sub>/gVS. The observed trend can be attributed to the competing effects of solubilization and the formation of refractory compounds. At lower temperatures (170 and 180 °C), HTC effectively enhances biodegradability by solubilizing complex biopolymers and lysing microbial cells, thus increasing substrate bioavailability. However, the subsequent decline in biodegradability at 200 °C indicates that at higher temperatures, desirable intermediates are increasingly converted into recalcitrant products through Maillard reactions. This is consistent with the findings of [20], who demonstrated that Maillard reactions during HTC lead to recalcitrant compounds, and that their formation raises by increasing temperature and holding time.

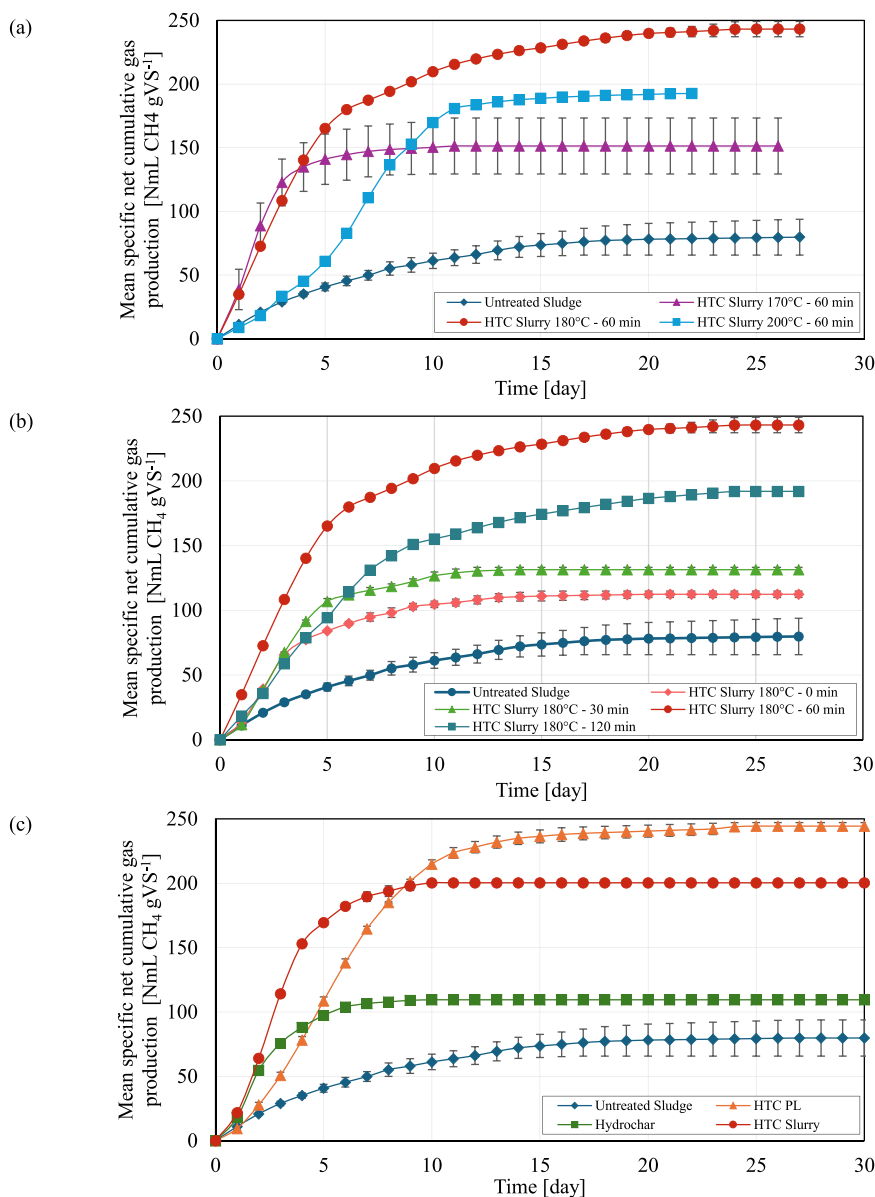
Following this, the effect of HTC holding time at 180 °C was investigated, comparing values of 0, 30, 60, and 120 min (Fig. 1b). The highest BMP values were observed for a holding time of 60 min. Extending the duration to 120 min resulted in a decrease in BMP compared to the 60-minute treatment, suggesting that prolonged exposure at this temperature might lead to the formation of inhibitory compounds or the degradation of readily biodegradable intermediates into more recalcitrant structures, negatively impacting overall methane yield. Based on these findings, HTC pretreatment at 180 °C for 60 min was identified as the optimal condition for maximizing waste sludge biodegradability in this study.

A final set of BMP tests was then conducted to evaluate the biodegradability of the individual product fractions generated under these optimal conditions: untreated waste sludge, HTC slurry, HTC PL, and hydrochar (Fig. 1c). Since the biogas yield (i.e., the asymptotic value of the BMP curve) serves as an indicator of anaerobic biodegradability, these findings highlight the overall effectiveness of the selected HTC treatment. Notably, the HTC PL exhibited the highest BMP with 206 % increase compared to the untreated waste sludge. The HTC slurry, representing the combined solid and liquid fractions, also demonstrated a high BMP value of 200.3 NmL CH<sub>4</sub>/gVS, 151 % increase over the untreated waste sludge. Finally, even the separated hydrochar fraction showed a higher BMP than the untreated waste sludge (37 % increase). It should be noted that the BMP for the HTC slurry in this test was lower than that recorded during the temperature optimization (243.2 NmL CH<sub>4</sub>/gVS). This variability is likely attributed to differences in the raw waste sludge batches used for each experimental set, highlighting the influence of feedstock characteristics on process outcomes. The hydrochar higher BMP relative to untreated waste sludge is a particularly striking result, especially given that this solid fraction contains approximately 55 % of the initial waste sludge COD, as detailed in the mass balance analysis in Section 3.2.5. These findings indicate that HTC primary carbon product, i.e. the hydrochar stream, remains largely biodegradable and biochemically available for further valorization. These outcomes are consistent with literature for similar HTC treatment conditions [8,14,19].

#### 3.2. Acidogenic fermentation tests

##### 3.2.1. Feedstock bioconversion

Following the optimization of HTC conditions, continuous



**Fig. 1.** Results of BMP tests (avg.  $\pm$  st.dev.): (a) comparison of untreated waste sludge and HTC slurry samples at different temperatures (170, 180 and 200 °C); (b) comparison of HTC slurry treated at 180 °C for different holding times (0, 30, 60 and 120 min), and (c) comparison of substrates (untreated waste sludge, HTC slurry, HTC PL and hydrochar) treated at 180 °C for a holding time of 60 min.

acidogenic fermentation tests were performed. CSTR1 and CSTR3 were fed with the whole HTC slurry, while SBR and CSTR2 were fed with the separated HTC PL. The characteristics of these feedstocks are summarized in Table 2. The characterization of these individual batches revealed that the primary carbon-related parameters (tCOD, sCOD, and

**Table 2**  
Characteristics (avg.  $\pm$  st.dev.) of HTC slurry and HTC PL used in the acidogenic fermentation tests for four process configurations.

		SBR (HTC PL)	CSTR1 (HTC slurry)	CSTR2 (HTC PL)	CSTR3 (HTC slurry)
tCOD	[gCOD/L]	21.5 $\pm$ 0.7	52.5 $\pm$ 6.2	25.7 $\pm$ 1.3	47.6 $\pm$ 6.1
sCOD	[gCOD/L]	21.5 $\pm$ 0.7	27.1 $\pm$ 3.3	25.7 $\pm$ 1.3	22.8 $\pm$ 2.4
N-NH <sub>4</sub> <sup>+</sup>	[mg/L]	178 $\pm$ 116	694 $\pm$ 233	611 $\pm$ 174	611 $\pm$ 174
P-PO <sub>4</sub> <sup>3-</sup>	[mg/L]	160 $\pm$ 59	205 $\pm$ 14	205 $\pm$ 14	205 $\pm$ 14
TS	[g/L]	21.6 $\pm$ 5.0	48.9 $\pm$ 6.6	16.8 $\pm$ 2.7	48.9 $\pm$ 6.6
VS	[g/L]	19.0 $\pm$ 4.0	30.6 $\pm$ 2.2	N.A.	29.0 $\pm$ 4.0
VFAs	[gCOD/L]	1.0 $\pm$ 0.1	0.6 $\pm$ 0.2	0.6 $\pm$ 0.2	0.6 $\pm$ 0.2

solids fractions) remained relatively consistent. This indicates a reproducible feedstock in terms of its organic content, which is the main driver for VFA production. The HTC PL feedstocks (for SBR and CSTR2) presented lower tCOD and TS values than the HTC slurry feedstocks (for CSTR1 and CSTR3), a direct result of the solid-liquid separation step that removes the particulate material (i.e., the hydrochar). A common characteristic across all feedstocks was the high concentration of soluble nutrients. These elevated ammonium and phosphate levels are a predictable consequence of subjecting waste activated (secondary) sludge, rich in microbial biomass, to hydrothermal conditions, which facilitates the thermal hydrolysis and deamination of proteins and the breakdown of nucleic acids, thereby liberating significant amounts of nitrogen and phosphorus into the aqueous phase, as demonstrated by [21] and [22]. Furthermore, the variations in the soluble nutrient concentrations among the different feedstocks directly reflect the use of multiple raw waste sludge batches with differing initial characteristics.

Fig. 2a displays the sCOD concentrations in the effluent of the four reactors throughout the experimental period, while the average values

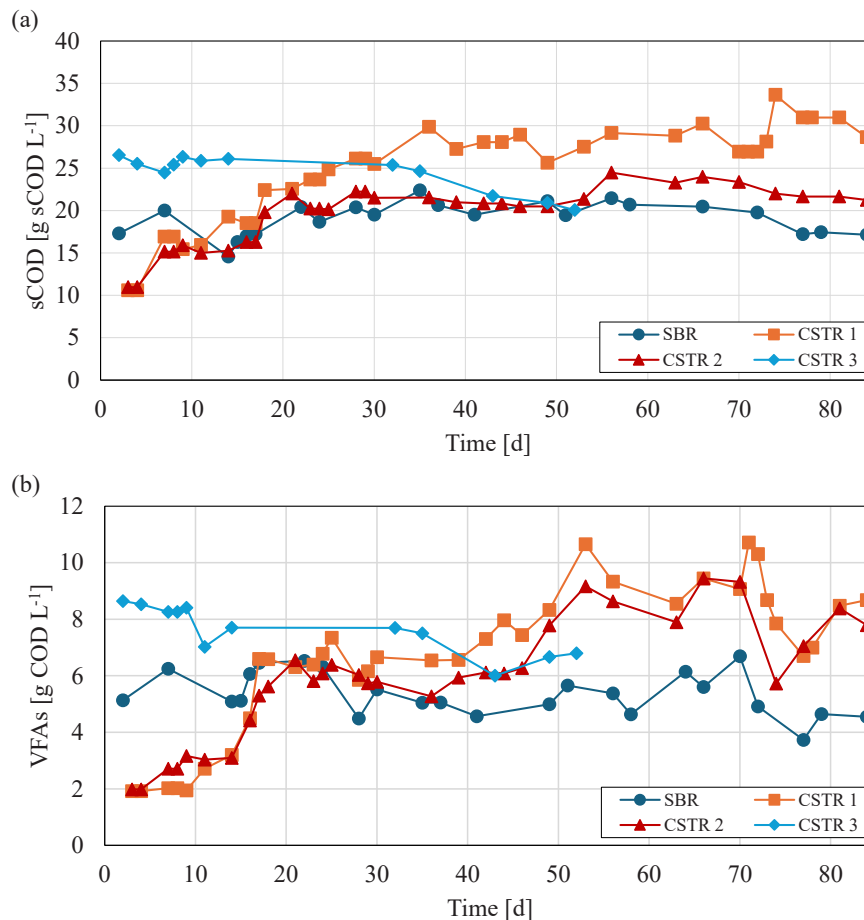


Fig. 2. Concentration trends of soluble COD (a) and VFAs (b) inside the effluent of the reactors used during fermentation tests.

are reported in Table 3. All reactors demonstrated relatively stable values after an initial acclimation phase, indicating consistent process performance despite variations in OLR due to changes in feedstock batches over the long experimental duration. Reactor CSTR1 exhibited the highest average effluent sCOD concentration. This can be attributed to its shorter HRT (4 d) compared to that of CSTR3 (8 d) and the fact that it was fed with the HTC slurry, which contained suspended solids potentially undergoing hydrolysis within the reactor, releasing soluble compounds. Beyond the influence of reactor configuration, the dynamic behavior of effluent sCOD must also be attributed to variations in the feedstock batches. The necessity of preparing fresh batches of HTC slurry and HTC PL throughout the experiment introduced a degree of variability in the influent COD concentrations. A direct consequence of this was that the reactors operated under pseudo-steady-state conditions at best, with effluent sCOD levels fluctuating in response to each change in feedstock.

Effluent total VFA concentrations generally followed sCOD trends (Fig. 2b), but VFA levels appeared more susceptible to changes in OLR and feedstock variability, potentially due to inconsistent solubilization of readily available organic carbon during HTC treatment. Statistical comparison using *t*-tests revealed that net VFA concentrations from reactors CSTR1 and CSTR2 were significantly higher than those from the SBR ( $p < 10^{-5}$  in both cases). However, no significant difference was observed when comparing VFA concentrations from CSTR1 and CSTR3 ( $p = 0.64$ ).

Nutrient release during fermentation was significant (trends available in Supplementary Material, Figure S1).  $N-NH_4^+$  concentrations (Table 3) increased approximately sixfold in the effluent compared to the influent after the acclimation period across all reactors (check Table 2 for reference). This indicates substantial hydrolysis of proteins and other nitrogenous organic compounds, likely enhanced by the HTC pretreatment, an observation also reported by [8] for fermented

Table 3

Effluent characteristics and performance indicators (avg.  $\pm$  st.dev.) for four process configurations obtained in the acidogenic fermentation tests at steady state.

		SBR	CSTR1	CSTR2	CSTR3
sCOD	[gCOD/L]	18.9 $\pm$ 1.7	27.2 $\pm$ 3.1	21.4 $\pm$ 1.6	24.4 $\pm$ 2.1
N-NH <sub>4</sub> <sup>+</sup>	[mg/L]	9967 $\pm$ 196	1894 $\pm$ 452	1714 $\pm$ 167	1560 $\pm$ 72
P-PO <sub>4</sub> <sup>3-</sup>	[mg/L]	208 $\pm$ 25	312 $\pm$ 125	231 $\pm$ 37	329 $\pm$ 113
TS	[g/L]	14.3 $\pm$ 1.8	51.1 $\pm$ 6.9	13.8 $\pm$ 1.6	56.8 $\pm$ 1.8
VS	[g/L]	11.9 $\pm$ 1.5	30.6 $\pm$ 2.2	3.3 $\pm$ 1.0	28 $\pm$ 0.1
VFAs	[gCOD/L]	5.6 $\pm$ 0.9	7.8 $\pm$ 1.4	6.8 $\pm$ 1.3	7.6 $\pm$ 0.8
pH	-	6.5 $\pm$ 0.3	6.7 $\pm$ 0.7	6.9 $\pm$ 0.5	6.5 $\pm$ 0.1
Y <sub>A</sub>	[%]	21.1 $\pm$ 4.2	25.7 $\pm$ 4.6	23.5 $\pm$ 4.4	30.6 $\pm$ 2.5
E <sub>A</sub>	[gCOD-VFA <sub>OUT</sub> / gCOD <sub>s,OUT</sub> ]	0.30 $\pm$ 0.06	0.29 $\pm$ 0.05	0.26 $\pm$ 0.04	0.31 $\pm$ 0.02
P	[gCOD-VFA <sub>OUT</sub> / (L*d)]	2.3 $\pm$ 0.5	1.7 $\pm$ 0.4	1.5 $\pm$ 0.3	0.9 $\pm$ 0.1

HTC-treated waste sludge. P-PO<sub>4</sub><sup>3-</sup> concentrations (Table 3) remained relatively constant in the SBR and CSTR2 (fed with HTC PL), whereas they increased more in CSTR1 and CSTR3 (fed with HTC slurry). This variability might be related to the hydrolysis of organic phosphorus present in the solid fraction of the HTC slurry feed.

No external pH control was applied during the fermentation tests. The reactors initial pH was approximately 8, equal to the one of the HTC-treated feedstocks. During the startup phase, the biological production of VFAs naturally acidified the reactor contents. This process drove the pH down until it stabilized in a mildly acidic range of 6.0–6.5 in all configurations, indicating a balance between VFA production and the system inherent buffering capacity. Average values and temporal trends are presented in Table 3 and Figure S1, respectively.

Average stabilized biogas production rates normalized by the volume and relative compositions were: 189 ± 63 mL/(d•L) for SBR (approx. 63 % CH<sub>4</sub>, 37 % CO<sub>2</sub>, 10 % H<sub>2</sub>, H<sub>2</sub> measured only here), 286 ± 108 mL/(d•L) for CSTR1 (approx. 40 % CH<sub>4</sub>, 60 % CO<sub>2</sub>), 447 ± 119 mL/(d•L) for CSTR2 (approx. 45 % CH<sub>4</sub>, 55 % CO<sub>2</sub>), and 206 ± 116 mL/(d•L) for CSTR3 (approx. 30 % CH<sub>4</sub>, 70 % CO<sub>2</sub>). Detailed biogas data are provided in the Supplementary Material, Figure S2 and Table S1. The presence of methane indicates that the methanogen inhibition strategy (inoculum heat treatment, initial BES addition) was not fully effective throughout the long-term operation, or that methanogens recovered. Despite this, the overall diversion of COD to methane was minor (1.4–9.1 % of influent COD, see Section 3.2.5), having a negligible impact on the final VFA yields. This outcome represents a more practically achievable state than complete methanogenic suppression, reflecting the performance of full-scale fermenters where a low background level of methane production is often observed.

The average VFA composition profiles at steady state were similar across all reactors (average values in Fig. 3 and time trends in Figure S3 of Supplementary Material), dominated by acetic acid, followed by propionic acid. This VFA spectrum is consistent with previous studies fermenting HTC-treated waste sludge [8,13]. Notably, longer-chain VFAs (butyric, valeric, caproic acids, including isomers) constituted a significant fraction of the total VFAs: 31 % in SBR, 37 % in CSTR1, 40 % in CSTR2, and 38 % in CSTR3. This high proportion of longer-chain VFAs is particularly advantageous if the fermentate is intended as a substrate for high-value biopolymer production, such as PHAs [23], thus highlighting a key potential valorization route for the process.

Mirroring the behavior of soluble COD, the TS concentrations within the reactors, while stable on average (Table 3), were subject to periodic fluctuations. This instability is directly attributable to the use of multiple feedstock batches throughout the experiment. As the TS content of the influent was never entirely consistent due to the variability in the raw WAS, the TS concentration inside each reactor closely tracked the characteristics of the most recently introduced feedstock batch.

### 3.2.2. Performance indicators

Performance indicators, calculated based on steady-state data, are shown in Table 3, being time trends reported in Figure S4 of Supplementary Material:

- **Acidification Yield:** the achieved average values surpass yields reported in several studies fermenting raw or thermally hydrolyzed waste sludge (e.g., 10 % by [24]; 14.1 % by [25]; 15.1 % by [13]), suggesting a beneficial effect from the HTC pretreatment. However, results are considerably lower than the ~59 % yields reported by [8] and [9] also using HTC-treated waste sludge, although [9] adopted an experimental approach based on batch tests that could have favoured VFA accumulation. CSTR3 achieved the significantly highest yield ( $p < 10^{-5}$  when compared to CSTR1), likely benefiting from its longer HRT (8 d), allowing more extensive conversion of influent sCOD. Comparing CSTR1 (HTC slurry feed) and CSTR2 (HTC PL feed), both with 4-day HRT, no statistically significant difference was found ( $p = 0.1$ ), implying that the presence of solids in the slurry might not contribute to VFA production over this timeframe. The similar yields of SBR (HRT of 4 d, SRT of 2 d) and CSTR2 (HRT = SRT = 4 d) suggest that decoupling HRT and SRT in the SBR while substantially increasing the OLR, did not offer a significant advantage in terms of overall conversion yield.
- **Acidification Efficiency:** relatively low values were obtained in all reactors, indicating that a substantial fraction of the soluble COD in the effluent consisted of non-VFA organic compounds, suggesting that much of the organic matter solubilized by HTC remained poorly fermentable or recalcitrant under these thermophilic acidogenic conditions. This aligns with observations from other studies on HTC process liquid fermentation [8,9,13]. The discrepancy between these moderate acidification yields and the higher overall biodegradability observed in the long-term BMP tests could be explained by the different SRT applied. The short SRT of the VFA fermenters, designed to enrich for fast-growing acidogens is likely too brief to retain the slow-growing specialist communities needed to degrade recalcitrant molecules [26]. The breakdown of complex compounds, such as the phenols and Maillard products generated during HTC, often requires syntrophic partnerships that are thermodynamically dependent on hydrogen scavengers and are slow to establish [27]. Consequently, these critical organisms were likely washed out from the fermenters, whereas the long SRT of the BMP test allowed for their retention and activity, leading to a more complete degradation of the feedstock.
- **VFA Productivity:** the SBR exhibited a significantly greater value ( $p < 10^{-5}$ ). This is due to its shorter HRT (2 d) and corresponding higher OLR, maximizing the rate of VFA generation per unit volume. This highlights the trade-off between productivity and conversion efficiency: shorter HRTs favour higher rates (productivity), while

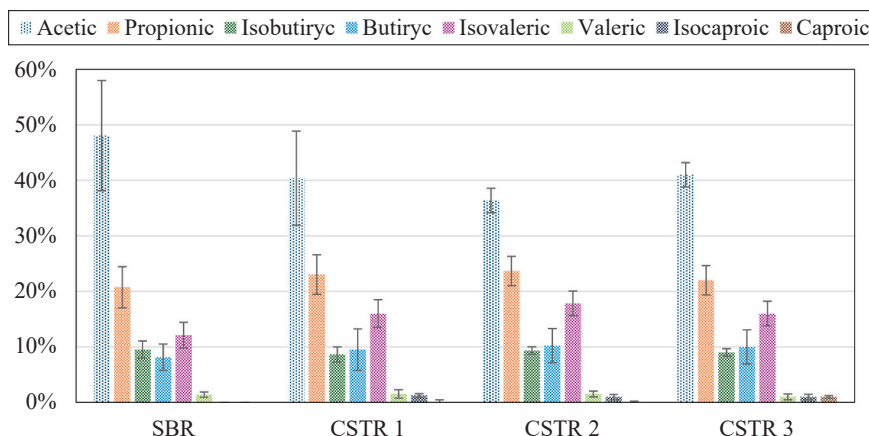


Fig. 3. VFA composition (avg. ± st.dev.) in the effluent of acidogenic fermentation tests at steady state.

longer HRTs allow for more complete conversion (higher yield and efficiency), as seen in CSTR3.

### 3.2.3. Microbial community characterization

To gain insight into the observed acidogenic fermentation performance, the microbial community structures in the inoculum and in the steady-state biomass of reactors CSTR1 (HTC slurry feed, HRT of 4 d) and SBR (HTC PL feed, HRT of 4 d, SRT of 2 d) were analyzed via 16S rRNA gene sequencing. Phylogenetic analyses revealed distinct community compositions between the two reactors (Fig. 4a). CSTR1 was heavily dominated by Proteobacteria (~ 70 % relative abundance), with Coprothermobacterota (~ 17 %) and Campilobacterota (~ 11 %) as

other major phyla. While Proteobacteria are common in fermentation systems and possess hydrolytic capabilities [28], such high dominance is notable, though observed by others [9,13]. As for Coprothermobacterota, they are characteristically thermophilic fermenters often found in high-temperature anaerobic environments [29], consistent with the 50 °C operating temperature. The presence of the phylum Campilobacterota (formerly Epsilonproteobacteria) in CSTR1 is significant, as a key metabolic feature of this group is the oxidation of reduced sulfur compounds, suggesting that biochemical pathways related to sulfur cycling were relevant within this reactor [30].

In contrast, the SBR exhibited a more diverse and evenly distributed community. While Proteobacteria (~ 22 %) and Coprothermobacterota

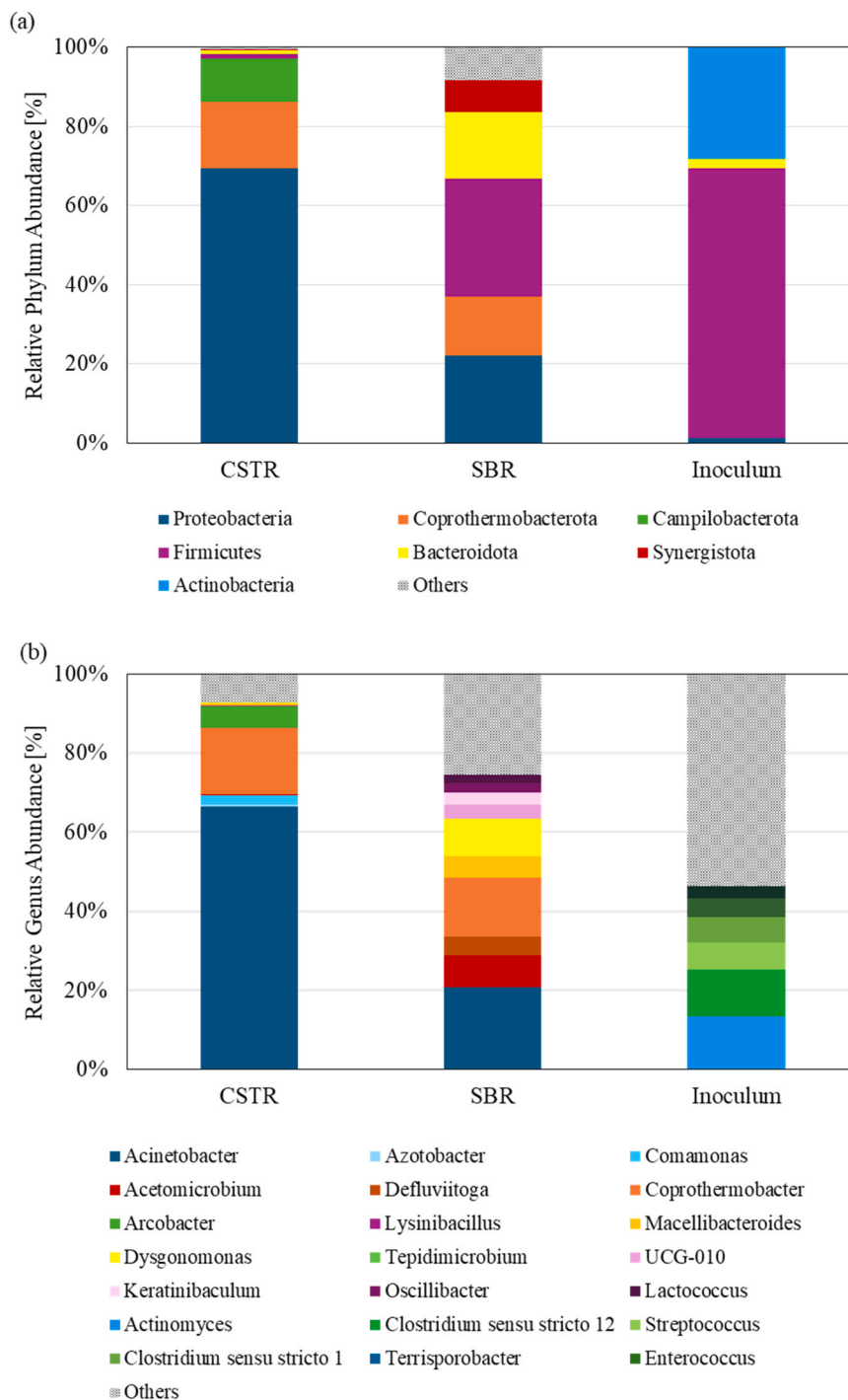


Fig. 4. Microbial community structures at phylum (a) and genus (b) level for reactors CSTR2, SBR and the common inoculum.

(~ 15 %) were still present, Firmicutes (~ 30 %), and Bacteroidota (~ 17 %), were also well represented phyla, with contributions from Synergistota and Actinobacteriota. The higher abundance of Firmicutes and Bacteroidota in the SBR is significant, as these phyla contain many well-

known fermentative bacteria responsible for hydrolysis and acidogenesis, producing VFAs from various substrates like proteins and carbohydrates [31–33]. The initial inoculum showed a higher relative abundance of Actinobacteria compared to the reactors, potentially due

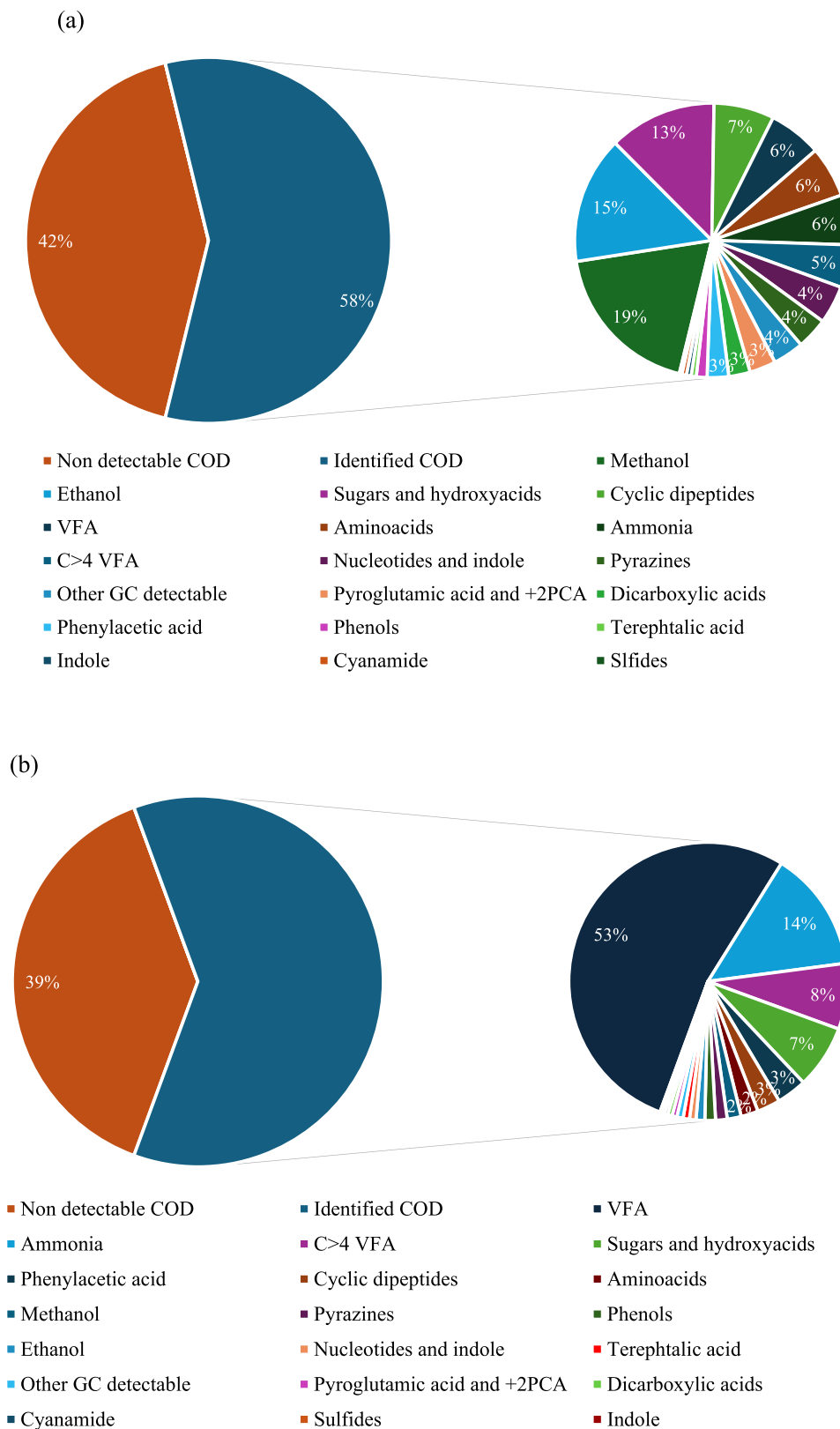


Fig. 5. Speciation of COD obtained through direct GC-MS analysis of (a) HTC PL and (b) effluent from reactor CSTR3. All data are presented as fraction of soluble COD.

to their known tolerance to humic substances [34] which might be present initially but selected against during reactor operation focused on VFA production.

At the genus level (Fig. 4b), the differences were even more pronounced. CSTR1 was overwhelmingly dominated by *Acinetobacter* (> 60 %), followed by *Coprothermobacter* (~ 17 %) and *Arcobacter* (~ 5 %). The high abundance of *Acinetobacter*, often considered aerobic or facultative, in this strictly anaerobic, thermophilic CSTR is not common, although its persistence in anaerobic systems has been reported [35]. Its presence cannot be attributed to survival through the pretreatment, as *Acinetobacter* does not form spores so that it could not withstand the high temperature and pressure of the HTC process [36]. Furthermore, the stable and continuous production of VFAs and biogas throughout the experiment confirms the maintenance of strictly anaerobic conditions, ruling out significant oxygen intrusion as a factor for its growth. Therefore, its dominance more likely reflects a unique metabolic adaptation to the substrate provided. A key explanation for its success lies in its well-documented capability as a polyphosphate-accumulating organism (PAO). Certain PAOs can utilize their internal stores of polyphosphate to generate energy for the anaerobic uptake and storage of simple organic compounds, such as the VFAs abundant in the fermenter [37]. Given the high phosphate concentrations in the feedstock, this metabolic strategy would allow *Acinetobacter* to thrive and efficiently sequester carbon even in the absence of external electron acceptors, likely explaining its high abundance in this VFA-rich environment. *Coprothermobacter* is a known thermophilic, anaerobic proteolytic genus producing acetate and  $H_2/CO_2$  [37,38].

The SBR displayed greater genus-level diversity. While *Acinetobacter* (~ 21 %) and *Coprothermobacter* (~ 15 %) were still prominent, their dominance was considerably reduced compared to CSTR1. Other abundant genera included *Dysgonomonas*, *Defluviitoga*, *Keratinibaculum*, and *Lactococcus*. This higher diversity in the SBR might be fostered by its operational mode (sequencing batch with settling phases), potentially imposing different selective pressures compared to the continuously mixed CSTR1 and possibly benefiting from the sludge retention mechanism ( $SRT > HRT$ ).

Overall, the distinct microbial communities likely reflect the different operating conditions (CSTR1 vs. SBR, HRT, SRT, feed type HTC slurry vs. HTC PL), influencing the specific fermentation pathways and potentially contributing to the observed differences in performance metrics.

### 3.2.4. Chemical speciation of substrates and reaction intermediates

Direct GC-MS analyses (Fig. 5) revealed that only a small fraction of the soluble COD was detected, mainly consisting of methanol, ethanol, and other low-molecular-weight oxygenates. However, silylation of the sample disclosed that more than 50 % of the soluble organics could be identified, providing deeper insight into the molecular composition of HTC PL. The liquid phase was found to contain a complex mixture of compounds, exceeding the separation capacity of a standard GC column, and thus appearing as a partially unresolved hump. The most relevant constituents were sugar derivatives formed through dehydration and rearrangement of saccharides, together with cyclic dipeptides [39], amino acids, and nucleotides. Fermentation markedly altered the detectable composition. Sugars, amino acids, and cyclic dipeptides were almost completely converted into VFAs, hydroxy acids (e.g., lactic acid and hydroxyacetic acid), and reductive deamination products (e.g., phenylacetic acid). In contrast, pyrazines, phenols, and (presumably) the undetectable fraction of organics appeared largely unaffected in quantitative terms. The undetectable compounds are likely either insufficiently volatile for GC-MS (e.g., high-molecular-weight species) or present at concentrations too low to be detected. A full characterization of this unidentified fraction would require a multi-platform analytical strategy, employing techniques such as Size Exclusion Chromatography (SEC) for high-molecular-weight species, comprehensive two-dimensional gas chromatography (GCxGC) to resolve co-eluting

compounds, and ultra-high-resolution mass spectrometry like FT-ICR MS to identify the thousands of unique molecular formulas present [40].

Based on this assumption, two main factors may underlie the incomplete conversion of soluble COD into VFAs. The first is the limited biodegradability of certain high-molecular-weight compounds (e.g., humin-like structures) under anaerobic conditions, a phenomenon previously reported for the aqueous phase of HTC [18]. The second, and perhaps more intriguing, is the inherently difficult conversion of the multitude of highly diluted constituents, as explained by the thermodynamic constraints of mixed-culture biotechnology [41]. Conversion of minor HTC-derived compounds (present at concentrations in the mg/L range) is thermodynamically less favourable than that of major constituents (typically at g/L levels), with an energy penalty of 0.1–1 kJ/kgCOD. Such a penalty is particularly detrimental for the biological conversion of more stable molecules (e.g., those containing furan or benzene rings), especially when targeting VFAs, whose formation is slightly less favourable thermodynamically than methane. This hypothesis is consistent with the observation that HTC-derived liquid is significantly more biodegradable under methanogenic conditions than under acidogenic ones.

### 3.2.5. Process mass balance

To evaluate the overall efficiency of integrating HTC pretreatment with acidogenic fermentation, COD mass balance was constructed for the different process configurations (Fig. 6). COD flows were normalized relative to the initial COD of untreated waste sludge fed to the HTC process ( $COD_0$ ).

As for the HTC process, a substantial portion of the initial waste sludge COD was solubilised (45.5 %), while the remaining particulate fraction was separated by the filterpress as hydrochar (54.5 %). This hydrochar-bound COD did not enter the fermentation reactors fed with HTC PL (SBR, CSTR2) and thus did not contribute to VFA production in those systems.

By comparing the VFA output of CSTR1 and CSTR2, operated at the same HRT, the impact of feeding the hydrochar is highlighted. Indeed, CSTR1 shows higher VFA productivity, though being fed with slightly lower amount of non-VFA sCOD (sCOD-other). This finding suggests that hydrochar positively contributes to VFA productivity. Coherently, the higher fermentation yield is observed with CSTR3, which is also fed on the HTC slurry, but allows longer HRT. COD losses to methane were relatively low (less than 10 %) in all tested process configurations, indicating the successful suppression of methanogenesis.

Nonetheless, the overall conversion of initial waste sludge COD into VFAs remained limited, leaving a large fraction of the COD either as recalcitrant soluble compounds in the effluent or as unconverted solids. This suggests that while HTC at 180 °C for 60 min improves biodegradability compared to untreated waste sludge, as suggested by the results of BMP tests, further optimization (of HTC pretreatment or acidogenic fermentation) or different downstream processes may be needed to maximize carbon recovery from the more persistent organic fractions generated or remaining after HTC.

Indeed, based on the BMP results (Section 3.1), both the HTC PL and the hydrochar fractions are characterized by good anaerobic degradability, which is only partly retrieved as VFAs in acidogenic fermentation. Thus, for improving the overall carbon recovery, anaerobic digestion of the non-fermented yet degradable organics to produce biomethane appears to be a viable valorization route.

## 3.3. Techno-economic and scale-up perspectives

A critical aspect for the real-world implementation of this technology is its economic viability, considering the OPEX associated with HTC. The primary challenges in scaling up the process are related to continuous operation, materials handling, and, most importantly, energy integration. At full scale, the sustainability of the process is fundamentally dependent on a sophisticated heat recovery strategy. A conceptual

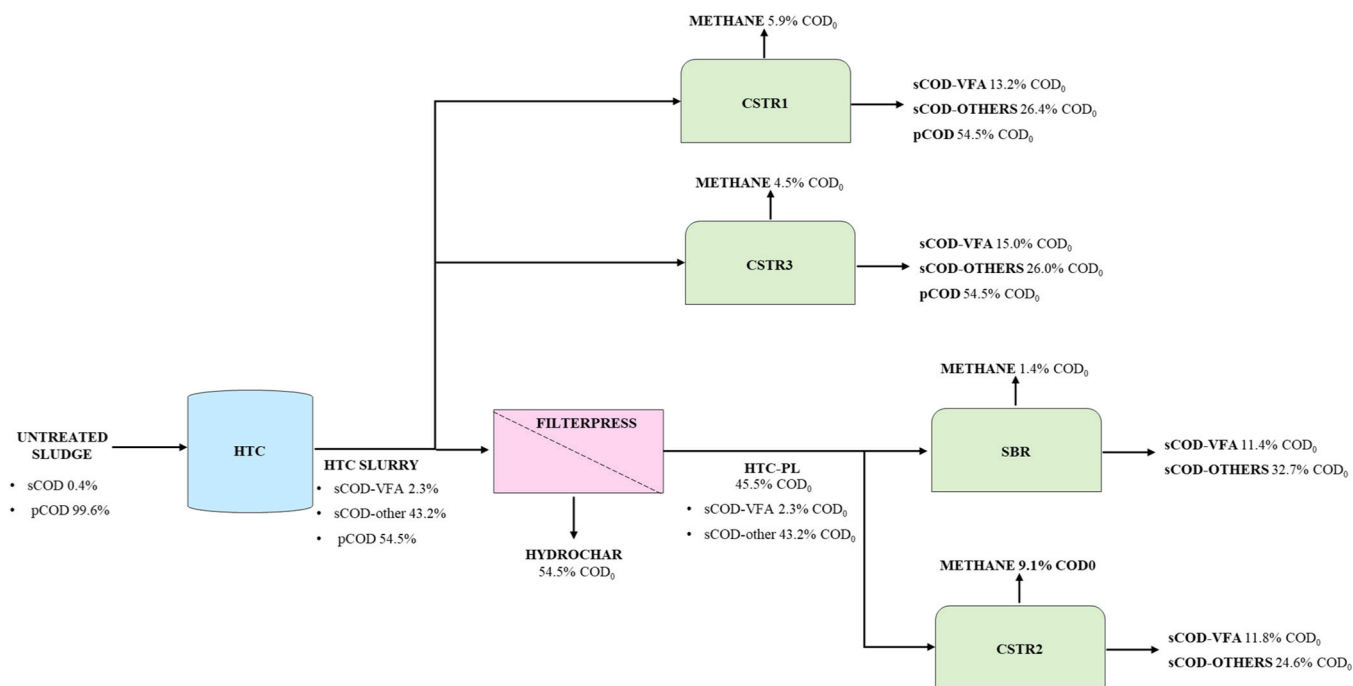


Fig. 6. COD mass balance of the four process configurations. 100 % COD<sub>0</sub> represents the total COD being fed to the HTC pretreatment as untreated waste sludge, pCOD is the COD associated with particulate components, sCOD-VFA is the sCOD associated with VFAs and sCOD-others indicates the non-VFA sCOD.

design would involve a primary heat exchanger where the hot HTC effluent pre-heats the incoming cold sludge, with residual heat then cascaded to maintain the thermophilic fermenter. The energy loop would be closed by utilizing the biogas produced from the anaerobic digestion of the hydrochar co-product to power the final trim heater and other plant equipment. This integrated design is essential to transform the process from an energy-intensive concept into a potentially energy-neutral or even energy-positive biorefinery.

From an economic standpoint, viewing HTC merely as an expensive "pretreatment" for fermentation is a mischaracterization of its function. A more accurate perspective is to frame the system as an integrated biorefinery that "cracks" a single waste stream into multiple value chains. The economic analysis must extend beyond the plant gate to include the substantial avoided costs. Based on a full-scale case study of 40,000 tons/year of waste sludge, the HTC process reduces the final mass of cake for disposal by 75–80 % and polyelectrolyte consumption by 90 %. This results in a 50–70 % saving on the overall operational costs of the sludge line, covering the process from the exit of the aerobic tank to the final destination of the dehydrated material. A major driver of this overall saving is the reduction in transport and disposal fees, which decrease proportionally with the 75–80 % mass reduction and represent the dominant (over 90 %) and most volatile cost in traditional sludge management. While OPEX for thermal energy, operator hours and maintenance is higher, the combined value from avoided disposal costs, reduced chemical consumption, internal energy recovery from the hydrochar, and the generation of high-value VFAs (suitable for biopolymer synthesis) inverts the economic balance. This approach makes the process not only sustainable but also profitable, with an estimated payback period of 3–6 years. This demonstrates that the high initial investment is a strategic one, unlocking a feasible and resilient pathway for waste sludge valorization.

### 3.4. Outcome discussion and practical implications

HTC pretreatment significantly improved the anaerobic biodegradability of the waste sludge, with both the resulting HTC PL and hydrochar fractions exhibiting potential for biogas generation. As for

acidogenic fermentation, despite the successful VFA generation, the overall conversion efficiency was moderate, with acidification yields reaching up to 0.30 gCOD-VFA<sub>OUT</sub>/gCOD<sub>IN</sub>, acidification efficiencies peaking around 0.31 gCOD-VFA<sub>OUT</sub>/gCOD<sub>S,OUT</sub> and volumetric productivities achieving 2.25 gCOD-VFA<sub>OUT</sub>/(L·d). This discrepancy between potential and actual anaerobic bioconversion underscores a fundamental limitation: the persistence of recalcitrant organic compounds generated or remaining after HTC, which are poorly degraded under acidogenic fermentation conditions, most likely in relation to the reduced HRT with respect to anaerobic digestion. The chemical speciation analysis highlighted that although fermentable substrates such as sugars and amino acids were effectively metabolized, a substantial portion of organics, including phenolic compounds, pyrazines, and complex Maillard reaction products, remained largely unconverted. This outcome is directly linked to the necessary trade-offs in selecting the HTC pretreatment conditions. While a higher process intensity (e.g., higher temperature or longer duration) might be expected to increase decomposition rates, our own optimization demonstrated that this is counterproductive for creating a biodegradable feedstock. We found that increasing the temperature to 200 °C or extending the holding time to 120 min resulted in a net decrease in anaerobic biodegradability. This confirms that the optimization of a pretreatment for a biological process is not governed by simple decomposition kinetics, but by the critical need to balance the desirable reactions of hydrolysis and solubilization against the detrimental formation of inhibitory and recalcitrant compounds [20,42]. Therefore, the moderate conversion efficiency observed is a consequence of operating at the experimentally determined "sweet spot" (180 °C, 60 min), which maximizes substrate bioavailability while inherently accepting the presence of a residual recalcitrant fraction. Overcoming this bottleneck is essential to unlock the full potential of HTC-assisted acidogenic fermentation. Future efforts could target tailored HTC conditions (e.g., shorter holding time, temperature adjustment) or implement complementary treatments (e.g., advanced oxidation, enzymatic hydrolysis) to depolymerize or detoxify persistent molecules. A key challenge in degrading these compounds is retaining the necessary slow-growing microorganisms. Our attempt to achieve this by decoupling the SRT from the HRT in the SBR configuration was

hindered by poor sludge settleability, preventing effective biomass retention. Furthermore, simply doubling the SRT to 8 days in the CSTR3 reactor yielded only a marginal improvement in conversion efficiency, suggesting that much longer solids retention times may be required, which in turn increases the risk of promoting methanogenesis. Variations in waste sludge characteristics should also be carefully accounted for, as they may significantly influence the results, with this aspect emerging from literature comparison.

The multi-faceted experimental design allowed for a clear deconstruction of how different operational variables influenced process outcomes. The direct comparison of CSTR1 (HTC slurry) and CSTR2 (HTC PL) under identical hydraulic conditions revealed that the presence of the hydrochar had a minimal impact on final VFA. The influence of retention time was clearly demonstrated by comparing CSTR1 (4-day HRT) and CSTR3 (8-day HRT), which confirmed a critical process trade-off: longer HRT favoured higher conversion efficiency, whereas shorter HRT maximized volumetric productivity. This highlights the importance of selecting an HRT based on the desired process goal, whether it be maximizing conversion or output rate. Finally, the comparison between CSTR and SBR configurations, though complicated by challenges in maintaining the target SRT, underscored the profound impact of operational mode on microbial ecology. In particular, the SBR-based process configuration developed a more diverse and functionally redundant community associated with broader hydrolytic and acidogenic capabilities, with possible significant implications for process stability and adaptability, particularly in systems treating variable feedstocks.

The COD mass balance underscored the importance of integrating the downstream processing of the hydrochar, since approximately 55 % of the initial COD was retained in this fraction. The high anaerobic biodegradability of this hydrochar confirms its potential as a significant bioenergy source, which is critical for the overall process sustainability as discussed in detail in Section 3.3. However, it is also important to note that this high biodegradability, while beneficial for energy recovery, means the carbon in the hydrochar cannot be considered stable, limiting its potential for applications where long-term carbon sequestration is the goal. The combined application of acidogenic fermentation of the liquid and anaerobic digestion of the solid HTC products within the same biorefinery layout therefore represents a promising solution. Finally, from a system integration perspective, the generation of VFAs enriched in longer-chain compounds through HTC pretreatment should be carefully considered, as these are particularly well-suited for downstream biopolymer production, offering a viable pathway to transform waste sludge into high-value bio-based products.

#### 4. Conclusions

This study provided a comprehensive evaluation of an integrated HTC-fermentation process for waste sludge valorization. In addressing the primary research objectives, several key conclusions were drawn.

Firstly, the study successfully identified an optimal HTC pretreatment condition (180 °C, 60 min) that maximized the anaerobic biodegradability of the waste sludge, demonstrating a critical trade-off where higher severities became counterproductive due to the formation of recalcitrant compounds. Secondly, the continuous fermentation of this optimally treated feedstock generated a valuable VFA mixture enriched in longer-chain acids, but the process exhibited moderate conversion efficiency (~30 %). The multi-faceted experimental design revealed that while a longer HRT slightly improved efficiency, the primary limitation was the persistence of a recalcitrant organic fraction. Finally, the COD mass balance provided a crucial insight for practical implementation: most of the initial carbon (~55 %) is retained in the highly biodegradable hydrochar.

In conclusion, this work demonstrates that while the HTC-fermentation pathway is a viable and promising route for producing high-value VFAs, its overall sustainability is fundamentally dependent on an integrated biorefinery approach that also valorizes the energy-rich

hydrochar co-product. Future research should focus on strategies to improve the biodegradation of the persistent compounds and on the techno-economic optimization of this double output system. Furthermore, advancing this field will require the development of more standardized analytical protocols, both for assessing the VFA production potential of pre-treated substrates and for comprehensively characterizing the complex feedstock composition to better understand and predict bioconversion performance.

#### CRediT authorship contribution statement

**Marco Pesenti:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Maria Jose Hernandez Sanchez:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Elena Ficara:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Andrea Turolla:** Writing – review & editing, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Matteo Grana:** Writing – review & editing, Methodology, Formal analysis. **Cristian Torri:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alisar Kiwan:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniele Pirini:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation.

#### Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author used correction tools based on generative AI to improve text fluency. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

#### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marco Pesenti reports financial support was provided by ALFA Varese S.r.l. Marco Pesenti reports financial support was provided by Italian Ministry of University and Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jece.2025.120920](https://doi.org/10.1016/j.jece.2025.120920).

#### Data Availability

Data will be made available on request.

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