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Challenging the conductive paradigm: The unexpected role of sand in anaerobic digestion

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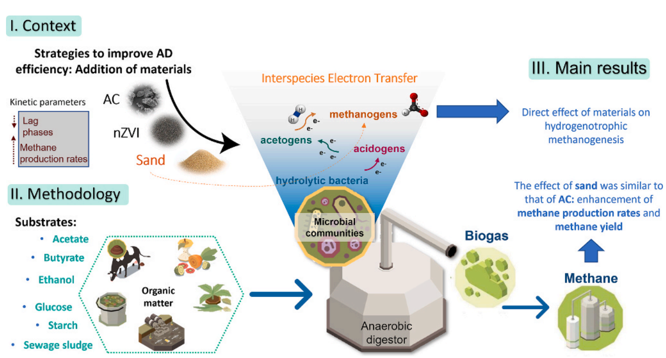
HIGHLIGHTS

- AC, nZVI and sand enhanced methane potential from several substrates.
- Sewage sludge degradation to CH_4 was significantly enhanced in the presence of sand.
- Sand outperformed conductive materials in MPR by pure cultures of methanogens.

GRAPHICAL ABSTRACT



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ABSTRACT

The application of conductive materials (CM) has emerged as a strategy to enhance methane production (MP) in anaerobic digestion (AD) systems. This study highlights the potential of sand as an alternative to CM for biomethane production during AD, utilizing complex microbial communities and pure cultures of methanogens. Sand (non-CM) was compared with activated carbon (AC) and nano-zero valent iron (nZVI), both CM, regarding MP kinetic parameters, including lag phase duration, methane production rate (MPR), and maximum MP. Biomethane potential tests revealed that all tested materials improved methane potential, with sand showing significant advancements in the degradation of various substrates, including sewage sludge, glucose, butyrate, and ethanol. Notably, sand led to an increase of up to 16.8 % in the maximum MP from sludge degradation compared to controls without materials, outperforming AC and nZVI. In assays with pure cultures, sand increased the MPR from the hydrogenotrophic *Methanobacterium formicicum* culture from 9.87 ± 1.97 $mM{\cdot}d^{-1}$ to 12.45 ± 2.63 $mM{\cdot}d^{-1}$. No significant effect was observed on MPR from acetoclastic *Methanothrix harundinacea*. Our findings

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emphasize the remarkable potential of sand as a low-cost and environmentally friendly alternative to traditional CM to enhance AD efficiency.

1. Introduction

In engineered systems, anaerobic digestion (AD) represents a sustainable biotechnological process that converts solid organic and domestic wastes, and industrial wastewater into biomethane (CH_4), driven by the synergistic activity of anaerobic microbes. This biotechnological process is crucial for renewable energy production and is key to mitigating climate change. The interplay of microbial syntrophy relies on interspecies electron transfer through hydrogen/formate (IHT/IFT) mechanisms, as the overall thermodynamics depend on the ability of microbial communities to maintain low hydrogen partial pressures (Park et al., 2018). When the hydrogen partial pressure is sufficiently low ($<10^{-4}$ atm), H^+ can oxidize NADH, $FADH_2$, and other electron carriers to produce H_2 during bacterial metabolism, which is a complex and energetically demanding process (Li et al., 2024). In addition to thermodynamic constraints, the low diffusion rate of hydrogen molecules further limits the efficiency of IHT. As a result, slow organic degradation rates and low methane production (MP) yield characterize AD as a low-efficiency and energy-limited process (Elsayed et al., 2024).

During the last years, direct interspecies electron transfer (DIET) through conductive materials (CM) has been reported, where CM (e.g., activated carbon (AC)) serves as an electric conductive bridge between bacteria and methanogens (Lee and Lee, 2019; Kong and Zhang, 2023). This mechanism is analogous to IHT/IFT, but electron transfer between microorganisms occurs without using hydrogen or formate as electron shuttles. Numerous studies have highlighted the benefits of adding CM to AD, reporting increased MP and biogas yields and shifts in microbial community composition due to improved mass transfer (both electron and proton transfer) (Li et al., 2024).

While these findings have demonstrated improvements in methanogenesis driven by CM, the exact underlying mechanisms remain unclear, particularly the individual contributions of various AD performance stimulatory mechanisms (Braga et al., 2024; Li et al., 2024; Martins et al., 2018). Other stimulatory mechanisms have also been proven to enhance MP in both CM and non-CM-supplemented AD systems. For instance, the incorporation of particles or materials enhances biomass retention in digesters by providing additional surface area and biocompatibility, while specific pore structures promote microbial attachment and interactions for efficient CH_4 bioconversion (Baek et al., 2021; Langxian et al., 2024). Interestingly, Braga et al. (2024) demonstrated that non-CMs, such as glass beads and sand, have effectively accelerated methanogenesis in pure cultures, and zeolites (Zeo) exert effects that are similar, or superior, to those of carbon-based materials, which possess higher conductivity. Beyond enhancing methane production, the introduction of sand into anaerobic digestion systems may offer additional operational benefits, such as improved sludge dewatering, enhanced solubilization and volatile fatty acid production, and increased process stability (Jiang et al., 2021). However, potential challenges, such as grit accumulation, equipment wear, or the need for post-treatment separation, must be considered to ensure the sustainable integration of this process into full-scale operations. In addition, sand particles are normally associated with operational problems such as reactor clogging and abrasive effects, which can lead to increased wear on mechanical components (stirrers, pumps) of AD reactors (Rocamora et al., 2020; Karthikeyan and Visvanathan, 2013).

Given these findings and the existing knowledge gap regarding AD systems amended with different materials, this work aims to explore the potential of sand, as a non-CM, to enhance AD performance. Sand (non-CM) was directly compared with widely studied CMs, namely AC and nano-zero valent iron (nZVI), regarding their effect on the degradation of a range of mixed organic compounds, including mixed sewage sludge, starch, glucose, ethanol, butyrate, and acetate, with complex anaerobic microbial consortia. AC represents carbon-based passive conduction through surface-mediated DIET (Wang et al., 2020, 2021), while nZVI exemplifies metal-based redox mediation, promoting methanogenesis via hydrogen release and electron shuttling (Contreras-Dávila et al., 2021). Their selection allowed us to contrast these active materials with the inert behavior of sand. As a control experiment, pure cultures of hydrogenotrophic and acetoclastic methanogenic archaea were tested as well to demonstrate the impact of non-CM on H_2/CO_2 and acetate degradation, respectively. This study provides new insights into advancing AD by introducing a low-cost and natural material that may serve as an alternative to synthetic CM additives, offering an innovative approach to enhancing process efficiency.

2. Materials and methods

2.1. Preparation of materials

The materials tested were nZVI, AC, and sand. nZVI (nanopowder, particle size 50 nm, average surface area 20–25 $m^2 \cdot g^{-1}$, $Fe^o > 80$ %, $pH_{zpc} = 8.0$–8.5) was obtained from NANOIRON Future Technology. Granular activated carbon (Norix ROX 0.8, pellets with a diameter of 0.8 mm and a length of 5 mm) was supplied by Norit. The sand was sourced from Apúlia Beach (Esposende, Portugal), selected for its prior characterization and successful application in the methanogenesis stage of anaerobic digestion (Braga et al., 2024), as well as its availability and suitability as a non-conductive, low-cost material for testing alternative supports. The sand used in the present study was washed multiple times with distilled water, and heated at 550 °C for two hours to remove organic contaminants (Braga et al., 2024). Granular activated carbon and sand samples were prepared as described in Braga et al. (2024). The AC was crushed to form a powder, and the materials (AC and sand) were sieved with particle sizes <280 μm. nZVI is then considered a nanosized material, while AC and sand are microsized materials. Table 1 summarizes the physicochemical characteristics of the materials. When not experimentally determined, the data were obtained from literature or provided by manufacturing companies.

2.2. Biomethane potential test

Biomethane potential tests (BMP) were employed to evaluate the effect of material addition on the profiles of substrate bioconversion to biomethane under the optimal conditions defined by Holliger et al. (2016). The digested sludge (inoculum) was periodically obtained from a wastewater treatment plant (WWTP) in northern Portugal, operated by Águas do Norte S.A. (Portugal), with a volatile solid (VS) content ranging between 1.0 % and 1.6 % (ww^{-1}). This WWTP was designed to serve a population equivalent of 49 891 inhabitants and treat an average daily flow rate of 7 600 $m^3 \cdot d^{-1}$. The main component of this WWTP is domestic wastewater, and the treatment system is divided into the liquid phase and the solid phase. The liquid phase consists of preliminary, secondary (two Carrousel reactors with 3 600 m^3 each), and tertiary treatment, while the solid phase consists of the anaerobic treatment (mesophilic anaerobic digester with 1 260 m^3 of working volume) of the sludge resulting from the process.

Anaerobic conditions were ensured by flushing pure N_2 into each bottle. Each experiment was carried out in triplicate. The presence of 500 $mg \cdot L^{-1}$ nZVI, AC, and sand was tested during the AD of substrates, including acetate, butyrate, ethanol, glucose, starch, and mixed sewage sludge. The fixed concentration of 500 $mg \cdot L^{-1}$ was selected based on previous optimization studies conducted by our research group. In

Salvador et al. (2017) different concentrations of carbon nanotubes (CNT) (0.1–5 g·L^{-1}) were tested in pure cultures of methanogens. Additional preliminary assays using this concentration range were conducted with AC, and the results confirmed 500 mg·L^{-1} as the optimal concentration (data not shown). This dose provided the best balance between enhanced methane production and reduced lag phase, without the inhibitory effects observed at higher concentrations. Mixed sewage sludge was also supplied by Águas do Norte S.A. (Portugal) and was composed of a mixture of primary and secondary sewage sludge (COD = 68.3 ± 9.3 g·L^{-1}, pH = 5.7 ± 0.3, TS = 8.198 ± 1.331 (%); VS = 5.595 ± 1.287 (%), VS/TS = 67.4 ± 6.3 %, where COD is the chemical oxygen demand, and TS and VS are the total and volatile solids content, respectively). The Automatic Methane Potential System (AMPTS II, BPC Instruments, Sweden) was used to conduct the BMP experiments until each bottle achieve a negligible CH_4 production (<5 mL·d^{-1}), at a mesophilic temperature range (~37 °C) and under intermittent conditions (1 min every 10 min) at 120 rpm. Substrate concentrations were fixed at 4 gCOD·L^{-1}, aiming to achieve the theoretical value of 560 mL of CH_4 (assuming that 1 gCOD results in 350 mL of CH_4 at PTN) after complete digestion in a working volume of 400 mL. COD, and total and volatile solids are registered in Table 2. The inoculum substrate ratios (g·g^{-1} VS) were 2.1, 5.2, 5.0, 4.2, 3.6, and 6.2 for acetate, butyrate, ethanol, glucose, starch, and sludge, respectively. Blank assays containing only the inoculum were carried out to measure endogenous methane production. For each tested material (nZVI, AC and sand), a control assay with only the substrate under study (acetate, butyrate, ethanol, glucose, starch, and mixed sewage sludge) was prepared as well. This allowed us to isolate the effect of each material on methane production.

Table 2
Substrate and inoculum composition for BMP tests.

Substrate	g COD ·g$^{-1}_{substrate}$	g COD · g^{-1} inoculum	g TS · g^{-1} inoculum	g VS · g^{-1} inoculum
Acetate	0.683	1.602 ± 0.002	2.01 ± 0.02	1.27 ± 0.03
Butyrate	1.381	1.599 ± 0.001	2.33 ± 0.39	1.56 ± 0.16
Ethanol	2.083	1.599 ± 0.000	1.53 ± 0.01	0.99 ± 0.01
Glucose	0.970	1.603 ± 0.002	2.39 ± 0.02	1.64 ± 0.02
Starch	0.990	1.601 ± 0.003	2.24 ± 0.02	1.51 ± 0.02
Sludge	0.109	1.599 ± 0.002	2.43 ± 0.01	1.49 ± 0.01

2.3. Pure culture incubation conditions

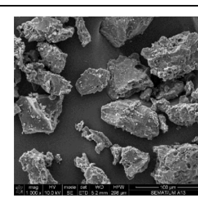
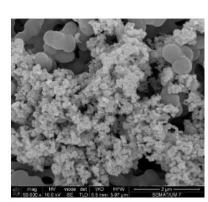
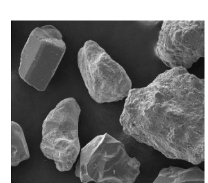
Two methanogens, one hydrogenotrophic (*Methanobacterium formicicum* (DSM 1535 T)), and one acetoclastic (*Methanothrix harundinacea* (DSM 17206 T)) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and maintained in our laboratory. The methanogens were cultivated under strict anaerobic conditions in saline bicarbonate-buffered mineral medium, supplemented with a cocktail of vitamins as described elsewhere (Stams et al., 1993).

The incubation assays with methanogens and the abiotic assays were carried out as described in Salvador et al. (2017). Abiotic assays were performed using a liquid substrate (acetate, 10 mmol·L^{-1}) or a gaseous substrate (H_2/CO_2, 80:20 %, 170 kPa). Except for the incubations with the gaseous substrate, the headspace of the bottles was flushed with N_2/CO_2 (80/20 % v/v, 170 kPa). All batch incubations were performed in 120 mL bottles, with working volumes of 55 mL for *M. formicicum* assays, and 62 mL for *M. harundinacea* incubations. In both biotic and abiotic assays, the experimental conditions consisted of a control without materials and those with the supplementation of nZVI, AC, and sand (at a concentration of 500 mg·L^{-1}). All assays were performed in triplicate. The bottles were protected from the light and incubated at 37 °C. *M. formicicum* assays were performed under agitation (120 rpm) to promote a better transport of H_2/CO_2 to the liquid phase. During abiotic assays, liquid samples were taken to monitor the oxidation–reduction potential (ORP), pH, and conductivity in the growth medium with and without materials.

2.4. Analytics methods

Gas samples from pure culture assays were collected from the incubation bottles by using a Pressure-Lock syringe (SGE Syringe, Trajan Scientific Australia Pty Ltd). Methane was measured in a gas

Table 1
Physicochemical characterization of materials regarding specific surface area (SBET), pH at zero-point charge (pHpzc), elemental analysis (values measured in weight, %) and SEM analysis of materials.

Materials	S_{BET}/ (m^2•g^{-1})	pH_{pzc}	C/ (%)	H/ (%)	N/ (%)	S/ (%)	Fe/ (%)	Si/ (%)	O/ (%)	SEM analysis	References
AC_Norit	1002	7.3	88.8	0.4	0.0	0.6	–	–	–		Braga et al., 2024
nZVI_NANOFER STAR	20–25	8.0 – 8.5	–	–	–	–	>80	–	–		Simsek and Turabik, 2017
Beach sand	0.05–0.1	9.5	–	–	–	–	–	33.8	51.7		Braga et al., 2024; Yang et al., 2016; Yao and Li, 2023

chromatograph-FID Chrompack 9000 (Shimadzu, Kyoto, Japan) with Carbowax 20 M column (2 m × 2 mm, 80–120 mesh), using the conditions described elsewhere (Duarte et al., 2020). Hydrogen measurements were performed using a BRUKER SCION 456 gas chromatograph (Billerica, MA), connected to a thermal conductivity detector and using a Molsieve packed column (13X 80/100, 2 m of length, 2.1 mm of internal diameter), with argon (30 mL·min^{-1}) as the carrier gas. Temperatures of the injector, column and detector were 100 °C, 35 °C and 130 °C, respectively. Acetate was analysed by high-performance liquid chromatography (Jasco, Tokyo, Japan) as described elsewhere (Duarte et al., 2020).

Scanning electron microscopy (SEM) imaging was performed using an FEI Nova 200 (FEG/SEM) microscope (FEI, Hillsboro, OR) at Materials Characterization Services of the University of Minho (SEMAT) (University of Minho, Guimarães, Portugal). Abiotic samples (nZVI, AC, and sand) and biotic samples (from pure cultures assays with and without materials) were prepared for SEM analyses as described by Salvador et al. (2017). The ORP and pH of the samples were measured using multiparameter analyzers, while the conductivity of the growth medium was determined using a Consort C3010 multi-parameter analyzer (Consort bvba, Turnhout, Belgium).

2.5. Mathematical and statistical analysis

The cumulative methane production data obtained in the BMP were fitted by the modified Gompertz equation (Eq. (1). as described by Sousa et al. (2013).

$$MP(t) = P \bullet \exp\left[-exp\left[\frac{Rm \bullet e}{P}(\lambda - t) + 1\right]\right] \tag{1}$$

where M(t) represents the cumulative methane production (mmol·L^{-1}), P is the maximum methane production (mmol·L^{-1}), Rm represents the methane production rate (mmol·L^{-1}·d^{-1}), e = 2.7182818, λ is the lag phase time (days).

Standard errors were calculated for each kinetic parameter, and the R^2 value was used to assess the fit of experimental data with the modified Gompertz model. The objective was to compare the effect of each material in accelerating the MPR from different substrates.

Statistical analysis for comparing kinetic parameters between different conditions was performed using Excel's two-way single-factor analysis of variance (ANOVA) followed by Tukey's post hoc test. The statistical differences were set at the $p < 0.05$ level.

3. Results

3.1. Biomethane potential tests

All tested materials demonstrated improved MP efficiency across all substrates compared to the control without materials (Table 3). Among them, sand showed the best experimental improvements, with increases in MP yield (CH_4 mL·gVS^{-1}) of 16.8 %, 10.9 %, 15.9 %, 11.3 %, 12.1 %, and 8.2 % in MP from sludge, starch, glucose, ethanol, butyrate, and acetate, respectively. The effect of sand was either slightly superior or comparable to that of AC (MP enhancements of 14.3 %, 3.7 %, 16.3 %, 11.4 %, 11.5 %, and 8.1 %, for the same substrates in the same order) and outperformed nZVI (MP enhancements of 7 %, 0.6 %, 9.5 %, 5.9 %, 7.8 %, and 3.0 %).

Table 3 shows the duration of the lag phase and the estimated MPR for all treatments with the substrates mentioned above. Sand consistently outperformed nZVI in enhancing MPR, achieving increases of 17.3 %, 37.4 %, 22.1 %, 29.8 %, 26.7 %, and 8.7 % compared to the control, for sludge, starch, glucose, ethanol, butyrate, and acetate, and achieved MPR improvements comparable to those observed with AC. While MPR and MP enhancements were determined with the addition of sand, no reduction in the lag phase was observed compared to the control without materials or to the other tested materials (Table 4).

3.2. Impact of sand, AC, and nZVI on methanogenic activity in pure cultures

Methanogenic activity assays using pure archaeal cultures amended with sand, AC, and nZVI revealed distinct effects on the MPR and lag phase duration for the hydrogenotrophic *M. formicicum* and the acetoclastic *M. harundinacea*. The kinetic parameters for both microorganisms are presented in Table 5. Among the tested materials, only sand enhanced MPR by *M. formicicum*, which increased from 9.87 ± 1.97 mM·d^{-1} in the control without materials to 12.45 ± 2.63 mM·d^{-1}. In contrast, AC and nZVI resulted in lower MPR values of 6.58 ± 0.63 mM·d^{-1} and 2.32 ± 0.36 mM·d^{-1}, respectively. For *M. harundinacea*, MPR in the control without materials was 0.46 ± 0.04 mM·d^{-1}. At the same time, the presence of nZVI, AC, and sand resulted in values of 0.35 ± 0.04 mM·d^{-1}, 0.31 ± 0.04 mM·d^{-1}, and 0.42 ± 0.02 mM·d^{-1}, respectively, indicating no significant improvement. Sand showed no inhibitory effect during the *M. harundinacea* conversion of acetate to CH_4. In contrast, AC showed a detrimental impact of 0.31 ± 0.04 mM·d^{-1} regarding the control treatment ($p < 0.05$). Concerning the reduction of lag phase duration in *M. formicicum*, the control exhibited a lag phase of 4.20 ± 0.38 days, whereas the nZVI, AC, and sand treatments reduced it to 1.27 ± 0.14 days, 2.34 ± 0.18 days, and 3.88 ± 0.00 days, respectively. Lag phase preceding MP was not observed in *M. harundinacea* pure culture. Abiotic control experiments revealed no significant variations in pH, ORP, or conductivity between AC and sand treatments, whereas nZVI increased the ORP from −325 mV to −224 mV. Additionally, hydrogen concentration in the nZVI abiotic experiment showed no significant variation (Fig. 1, supplementary information).

SEM analysis (Fig. 2) revealed distinct interactions between *M. formicicum* or *M. harundinacea* cells and the tested materials. Both AC and sand effectively functioned as growth supports for archaeal populations. In the analyzed spots (Fig. 2a-d), a high colonization of sand by microbial cells was observed. This suggests that sand may offer favorable surface properties for supporting cell proliferation. nZVI particles were observed to aggregate around *M. formicicum* cells, although no cell

Table 3
Experimental methane production (MP_E) efficiency from different substrates in assays amended with nZVI, AC, and sand. Increments are related to control assays (without materials).

Substrate	Control		nZVI			AC			Sand		
	MP_E [mL]	MP_E Yield [mL·gVS^{-1}]	MP_E [mL]	MP_E Yield [mL·gVS^{-1}]	Increment [%]	MP_E [mL]	MP_E Yield [mL·gVS^{-1}]	Increment [%]	MP_E [mL]	MP_E Yield [mL·gVS^{-1}]	Increment [%]
Sludge	236.4	42.0	273.2	48.7	13.7 %	275.5	49.0	14.3 %	**283.3**	**50.5**	**16.8 %**
Starch	465.2	79.5	468.1	79.9	0.6 %	483.6	82.5	3.7 %	**523.2**	**89.2**	**10.9 %**
Glucose	443.0	69.7	489.2	77.0	9.5 %	**489.2**	**83.4**	**16.3 %**	526.4	82.9	15.9 %
Ethanol	454.8	117.9	482.8	125.3	5.9 %	**512.8**	**133.1**	**11.4 %**	512.4	132.9	11.3 %
Butyrate	483.8	80.0	525.1	86.7	7.8 %	546.7	90.3	11.5 %	**550.4**	**91.0**	**12.1 %**
Acetate	545.8	110.3	562.7	113.7	3.0 %	594.0	120.0	8.1 %	**595.8**	**120.2**	**8.2 %**

In bold are highlighted the highest values for MP_E yield increment for each substrate.

Table 4
The Modified Gompertz parameters obtained from the BMP experimental results.

Substrate	Material	Lag phase [d]	$MPR_{máx}$ [$mL·d^{-1}$]	Increment	R^2
Sludge	None	not observed	125.2 ± 7.0 *a*	0.0 %	0.984 ± 0.006
	nZVI		143.1 ± 3.2*b*	14.3 %	0.976 ± 0.001
	AC		**147.3 ± 4.2*b***	**17.7 %**	**0.963** ± 0.004
	Sand		146.8 ± 6.2*b*	17.3 %	0.974 ± 0.002
Starch	None	0.661 ± 0.052 *a*	276.5 ± 15.1 *a*	0.0 %	0.997 ± 0.001
	nZVI	0.770 ± 0.016*b*	324.9 ± 4.2*b*	17.5 %	0.998 ± 0.000
	AC	0.743 ± 0.026 *a*	361.9 ± 19.7*b*	30.9 %	0.998 ± 0.000
	Sand	**0.756 ± 0.021*b***	**379.8 ± 16.4*b***	**37.4 %**	**0.999** ± 0.001
Glucose	None	0.111 ± 0.023 *a*	226.4 ± 16.6 *a*	0.0 %	0.992 ± 0.001
	nZVI	0.085 ± 0.011 *a*	255.9 ± 11.3 *a*	13.0 %	0.999 ± 0.000
	AC	**0.100 ± 0.016 *a***	**294.8 ± 12.0*b***	**30.2 %**	**0.999** ± 0.001
	Sand	0.083 ± 0.008 *a*	276.5 ± 2.2*b*	22.1 %	0.999 ± 0.001
Ethanol	None	1.064 ± 0.022 *a*	119.4 ± 20.9 *a*	0.0 %	0.992 ± 0.003
	nZVI	1.013 ± 0.039 *a*	144.2 ± 5.2 *a*	20.8 %	0.996 ± 0.000
	AC	0.931 ± 0.022*b*	151.2 ± 3.1 *a*	26.6 %	0.996 ± 0.000
	Sand	**1.056 ± 0.053 *a***	**155.0 ± 5.7*b***	**29.8 %**	**0.996** ± 0.000
Butyrate	None	0.850 ± 0.086 *a*	116.8 ± 20.5 *a*	0.0 %	0.996 ± 0.000
	nZVI	0.646 ± 0.023*b*	131.2 ± 4.5 *a*	12.3 %	0.997 ± 0.000
	AC	**0.744 ± 0.037 *a***	**159.8 ± 2.9*b***	**36.8 %**	**0.996** ± 0.000
	Sand	0.773 ± 0.004 *a*	148.0 ± 1.2*b*	26.7 %	0.997 ± 0.000
Acetate	None	0.245 ± 0.019 *a*	465.8 ± 2.8 *a*	0.0 %	0.995 ± 0.000
	nZVI	0.215 ± 0.006 *a*	475.1 ± 22.1 *a*	2.0 %	0.995 ± 0.000
	AC	0.217 ± 0.001 *a*	502.5 ± 1.9*b*	7.9 %	0.995 ± 0.000
	Sand	**0.235 ± 0.007 *a***	**506.3 ± 7.5*b***	**8.7 %**	**0.995** ± 0.000

Different lower-case letters indicate significant differences ($p < 0.05$); In bold are highlighted the higher values for $MPR_{máx}$ increment.

morphology or integrity changes were evident (Fig. 2d). In contrast, AC might have induced slight morphological changes in the cell structure (Fig. 2c). This observation was not further investigated, and because of that we cannot attribute this entirely to the presence of AC. Nevertheless, these observations highlight the differing impacts of each material on the development and spatial distribution of *M. formicicum* cells. Regarding *M. harundinacea*, nZVI formed aggregates around the cells without causing noticeable changes (Fig. 2f). Cell adherence was observed in the presence of AC, displaying apparent connections between adjacent microbial cells, suggesting that AC may promote microbial attachment and cell-to-cell interactions (Fig. 2g). Regarding granulometry, the sand used in this study was sieved to particles smaller than 280 μm, with the majority ranging from 50 to 150 μm based on sieve mesh specifications. The specific surface area of sand (0.05–0.1 $m^2·g^{-1}$) was obtained from literature and serves as a reasonable proxy for its particle size and surface accessibility, especially when compared to that of AC Norit (1002 $m^2·g^{-1}$) and nZVI (20–25 $m^2·g^{-1}$).

Table 5
MP kinetic parameters for *Methanobacterium formicicum* and *Methanothrix harundinacea* assays obtained with the Modified Gompertz model.

	Methanobacterium formicicum			*Methanothrix harundinacea*	
Material	MPR [$mM\ d^{-1}$]	λ [d]	R^2	MPR [$mM·d^{-1}$]	R^2
Control	9.87 ± 1.97 *ab*	4.20 ± 0.38 *a*	0.987 ± 0.005	0.46 ± 0.04 *a*	0.980 ± 0.011
nZVI	2.32 ± 0.36*c*	1.27 ± 0.14*c*	0.996 ± 0.002	0.35 ± 0.04 *ab*	0.979 ± 0.002
AC	6.58 ± 0.63*b*	2.34 ± 0.18*b*	0.990 ± 0005	0.31 ± 0.04*b*	0.958 ± 0.036
Sand	12.45 ± 2.63 *a*	3.88 ± 0.00 *a*	0.994 ± 0.000	0.42 ± 0.02 *a*	0.987 ± 0.002

Statistical differences between treatments were determined by two-way ANOVA, followed by Tukey's post hoc test with a significance level of $p < 0.05$. Different lower-case letters within the same column indicate statistically significant differences. Same letter – no difference; different letters – statistically different; combination of letters – intermediate group, with no significant difference with the groups whose letters it shares.

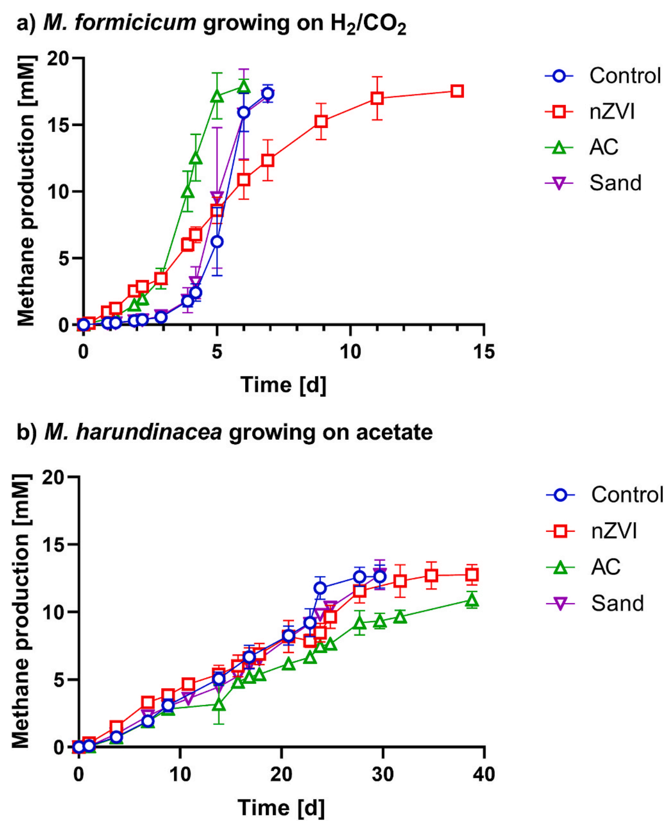


Fig. 1. Cumulative methane production profiles over incubation time for the following assays, with and without materials: a) *M. formicicum* growing on H_2/CO_2 and b) *M. harundinacea* growing on acetate. The results are presented as the average of triplicate assays, with the respective standard deviation.

4. Discussion

Extensive research cases have positioned CMs as key driving force in enhancing MP as a result of the electron transfer mechanism shift to DIET. Consistent with previous studies (Alam and Dhar, 2023; Jadhav et al., 2022; Kumar et al., 2021; Wang et al., 2016), our results showed that nZVI and AC as CMs yielded positive results in terms of methane production enhancement. Still, the exact mechanisms underlying the effects of these materials, unlike DIET facilitation, were not thoroughly understood. Applying sand in this study proved to be the most effective

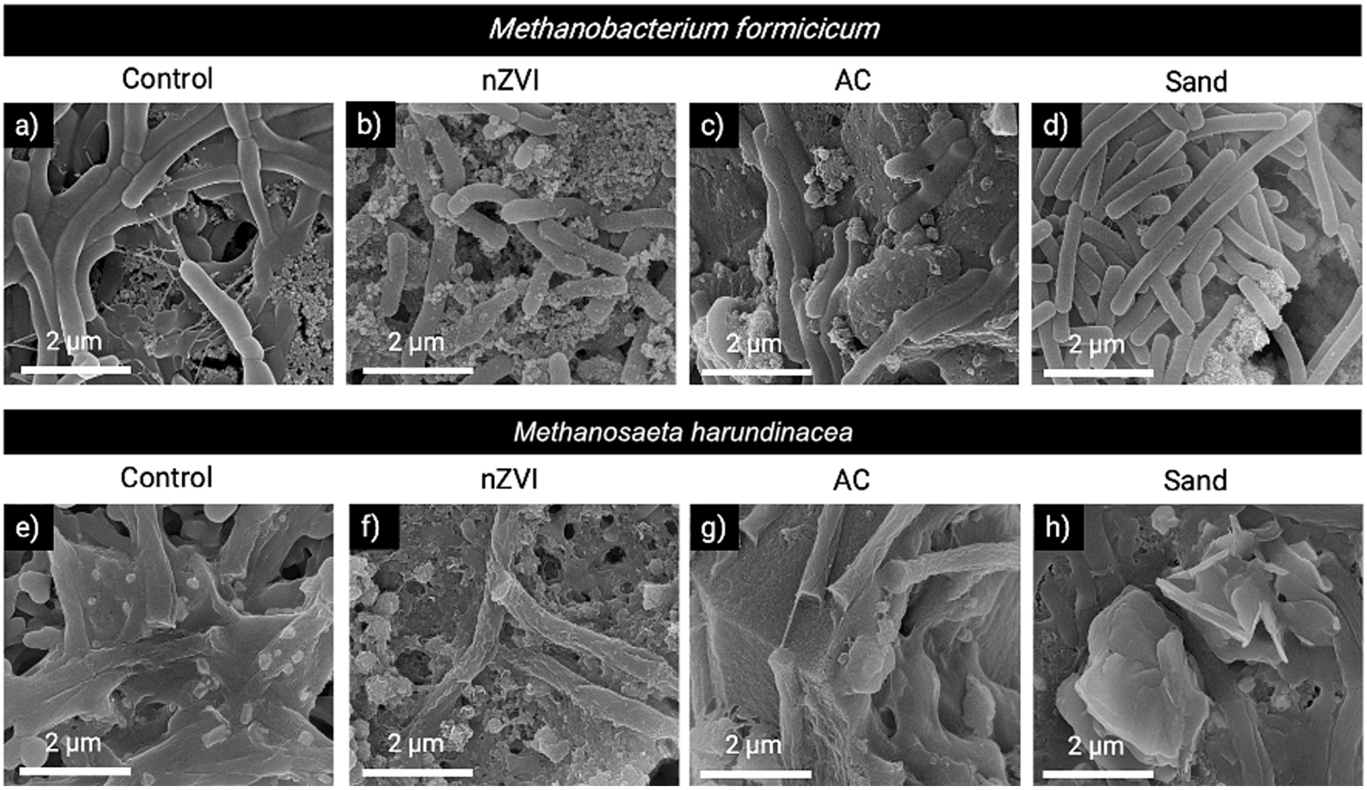


Fig. 2. SEM images showing the interaction between methanogens and materials (nZVI, AC, and Sand) in pure cultures of *M. formicicum* (a–d) and *M. harundinacea* (e–h).

treatment, significantly increasing MP yield and MPR across all tested substrates, including those typically more challenging to degrade, such as starch and sludge, rather than reducing lag phase duration. The impact of sand was particularly noticeable in sewage sludge degradation, significantly enhancing degradation by up to 16.8 % compared to the control without materials. This finding is significant as sludge degradation is often hindered by its complexity (Garrido-Baserba et al., 2015), and sand significantly improved the performance of the AD process. Our results demonstrate that the beneficial effects of sand were more pronounced when applied to a complex substrate, such as sludge and glucose, where a notable increase in methane production and degradation efficiency was observed. In contrast, for simpler substrates (e.g., acetate), no statistically significant differences were found between the amended and control groups. This suggests that sand's stimulatory role depends on substrate biodegradability and is especially important in conditions where hydrolysis and syntrophic cooperation are more limited. Interestingly, while AC is known for its high surface area and conductivity, the results of this study showed that sand, despite being a non-CM, outperformed AC in promoting MP across all tested substrates, suggesting that conductivity and surface area may not be the key factors influencing the improvement in AD performance. It raises the question of whether other properties of sand, such as its physical structure and composition, play a more significant role in enhancing the AD process than its electrical properties. Sand enhances microbial growth not by changing substrate energy needs, but by probably providing a stable surface for biofilm formation. The proof of that is that individual sand grains can support a highly diverse bacterial community, where just 4 to 8 single grains are sufficient to capture 50 % of species-level operational taxonomic units present in bulk sediments (Probandt et al., 2018). In addition, SEM analysis (Fig. 2d) showed that sand did not inhibit the growth of methanogens, especially in the *M. formicicum* assay. This physical support could facilitate microbial interactions, including syntrophic relationships through IHT/IFT (Akram et al., 2024; Poirier et al., 2017). Although mechanisms are still not well understood, sand's effectiveness aligns with findings that non-CM materials can promote microbial proliferation by encouraging biofilm development (Baek et al., 2021). While our study focused specifically on sand due to its availability, low cost, and prior characterization (Braga et al., 2024), we recognize that inert materials such as Zeo and glass beads have been studied, each with distinct outcomes. Importantly, not all non-CMs are beneficial. For example, Baek et al. (2021) evaluated various microplastics (High-Density Polyethylene, Polyethylene Terephthalate and Polyvinyl Chloride) and observed inconsistent or negative effects on methane production, depending on polymer type, concentration, and hydrophobicity. This highlights that chemical inertness alone does not guarantee a positive outcome; interactions with microbial communities and physicochemical properties (e.g., surface energy, porosity) are critical factors. Moreover, Braga et al. (2024) noted that non-CM materials can still contribute to methanogenesis through enhanced microbial growth and biofilm formation, even in the absence of conductivity. They also observed that silicon content was inversely related to the time needed for complete MP. While silicon is not essential for methanogens, it can accelerate the breakdown of organic matter and increase methane production, possibly by releasing bioavailable *ortho*-silicic acid that stimulates methanogenic activity. Its exact role in methanogenesis remains uncertain (Braga et al., 2024). Although SEM images of abiotic samples indicated that materials like sand and AC possess surface porosity and textural features that may favor microbial attachment (Table 1; Fig. 2), the correlation between these physical properties and microbial community structure remains speculative. Future studies should incorporate long-term studies and microbial-level analyses, such as 16S rRNA sequencing and biofilm-resolved SEM/EDS, to directly evaluate how these materials influence microbial colonization and syntrophic partnerships in anaerobic digestion systems.

In pure methanogenic cultures, methane pathways through acetate and H_2/CO_2 breakdown were analyzed without syntrophic interactions. Results show that sand had similar or better performance in MPR compared to all other treatments and controls without materials

(Table 5). SEM results also indicated that sand did not affect *M. formicicum* cell growth. Although statistical analysis showed no significant differences in MPR, assays for *M. formicicum* increased by about 21 % with sand, while no significant change was seen for *M. harundinacea*. This suggests that sand may create a more favorable microenvironment for hydrogen-utilizing methanogens, possibly due to better cell-surface interactions or biomass retention. While our study did not analyze syntrophic consortia or microbial community structure, this targeted stimulation of *M. formicicum* could be important in mixed cultures because increased activity of hydrogenotrophic methanogens might indirectly support syntrophic partnerships by maintaining low hydrogen partial pressure, thus stabilizing hydrogen thermodynamically and impacting overall AD performance. These results imply that sand promotes methanogenic biofilm development and improves H_2/CO_2 conversion to CH_4 by *M. formicicum*. Biofilm formation is vital for microbial communities, as it increases proximity between species and reduces mass transfer issues. Additionally, sand offers mechanical stability, helping biofilms withstand environmental stresses like water flow and shear forces (Tsagkari et al., 2022; Tang et al., 2023). This stability is key to maintaining biofilm integrity and supporting microbial interactions.

In *M. harundinacea* incubations, the sand did not enhance MPR compared to the control, whereas AC and nZVI had a slight negative effect on this kinetic parameter (Table 4). Despite that, cell proliferation and aggregation were observed in the presence of sand (Fig. 2h), while this was likely insufficient to influence acetate conversion. The MPR reduction observed with the CMs may be linked to interferences with extracellular polymeric substances (EPS) on microbial surfaces, hindering mass transfer due to alterations in the external matrix composition, as described by Li et al. (2019). In this regard, Nabi et al. (2024) highlighted that the effects of carbon-based composites on EPS are material-specific, meaning that even materials with similar carbon compositions can yield distinct EPS characteristics in AD systems. Such variability may impact microbial aggregation, biofilm formation, and nutrient transfer, ultimately influencing MPR kinetics.

Furthermore, our findings indicated limited enhancement of CM in MPR by pure cultures of methanogens. Remarkably, the pure cultures inocula of both methanogens used to prepare these experiments showed a high initial methanogenic activity, a condition that aligns with the main conclusions from Braga et al. (2025), who demonstrated that the microbial activity of the inoculum is a key factor influencing the impact of materials towards MP kinetics, i.e., the higher the initial activity, the lower the impact of the material on methane production. In their study, materials such as AC, Mag, and Zeo significantly reduced lag phase duration and enhanced MP in less active microbial communities, but showed minimal or no effect on highly active microbial communities or pure cultures. Although our current study did not analyze syntrophic consortia or microbial community structure, this selective stimulation of *M. formicicum* could be relevant in mixed cultures because an increase in hydrogenotrophic methanogens' activity may indirectly support syntrophic partnerships by maintaining low hydrogen partial pressure, thus facilitating hydrogen's thermodynamic stability, which impacts overall AD thermodynamics (Storck et al., 2016).

As noted, no significant changes were observed in bulk ORP or pH with sand present; however, microbial activity was consistently increased. This suggests that sand's stimulating effect may not be mediated through typical redox or acid-base shifts, but rather through indirect pathways (Wang et al., 2020). One possible explanation is the release of trace elements from the sand into the medium. Although not measured here, naturally occurring elements such as silicon, iron, or calcium found in mineral particles can leach in small amounts and affect microbial metabolism (Chen et al., 2016). Moreover, Braga et al. (2024) found a statistically strong correlation between the reduction of the total time required to achieve a complete methane production by *M. formicicum* and silicon content in Zeo, sand, glass beads, and AC, suggesting a role of silicon in methane production enhancement. Furthermore, the surface characteristics of sand might enhance microbial adhesion or cell clumping, potentially creating microscale zones that favor interspecies interactions or metabolite exchange, even without evident changes in bulk solution chemistry.

In summary, sand amendment had a greater impact on the conversion of complex substrates to methane and the activity of pure cultures of methanogens, in comparison to the amendments with AC and nZVI. In particular, the lower performance of nZVI on BMP and pure culture assays as CM could be due to nanomaterial aggregation, which can adversely affect microbial activity by disrupting cell membranes, promoting localized accumulation, and ultimately causing settling (Nguyen et al., 2018). This results in high local concentrations that smother active biomass or hinder substrate diffusion. Additionally, excessive aggregation may raise hydrogen partial pressure, a critical factor for syntrophic methanogenesis.

The use of sand as a non-traditional material offers significant advantages over conventional CM (Table 6). Sand is not only widely available and substantially less expensive (usually presents acquisition costs below 5 € per ton) (Islam et al, 2024), its extraction and processing also require less energy and results in lower greenhouse gas emissions compared to the production and disposal of synthetic or metal-based materials (Watari et al., 2023). The production of CMs entails energy-intensive processes, often involving high-temperature activation or synthesis under controlled conditions (Liang et al., 2021; Nguyen et al., 2021; Tian et al., 2023). Sand poses a negligible risk of toxicity or contaminant leaching and is easily separable by gravity. Meanwhile, CMs may require special handling (e.g., for nanomaterials), generate upstream emissions, and result in spent materials that may adsorb toxic substances and complicate disposal (Table 6). Taken together, these factors underscore the economic and environmental benefits of using sand as a sustainable support material, particularly when sourced from existing wastewater treatment infrastructure or natural deposits.

Nevertheless, sand extraction from nature could represent numerous environmental and social consequences (Bendixen et al., 2021). In the present study, and for reproducibility reasons, we used sand from a local beach. However, we envisage the use of sand recovered from the grit chamber of wastewater treatment plants (WWTP). Recently, this recovered sand is already being looked at as a valuable resource to be

Table 6
Lifecycle and economic analysis of sand vs conductive materials (Bruno et al., 2023; Islam et al., 2024; Liang et al., 2021; Nguyen et al., 2021; Tian et al., 2023; Watari et al., 2023).

	Sand	Conductive materials
Production and supply	Readily available globally as a low-value construction aggregate; Sourcing does not require specialised processing; Procurement costs are minimal	Requires energy-intensive manufacturing, special handling, and are often imported
Acquisition costs	< 5 € /ton	AC: 500 € to 2500 € per ton; nZVI: 2000 € to 10,000 € per ton; Biochar: 600 € to 2000 € per ton
Maintenance costs	Sand's abrasiveness can increase mechanical wear and equipment substitution	Finer particles can induce abrasion and clogging; Agglomeration issues, and corrosion (nZVI)
Handling needs	Basic separation	Waste management and Hazmat protocols
Environmental advantages	Minimal manufacturing footprint, requires only extraction, and for some uses a simple washing; No toxicity or leaching of harmful substances; Can be easily separated by gravity and recycled	Substantial upstream carbon and energy costs associated with production; Risk of nanomaterial release to the environment; Spent CM may contain adsorbed toxic compounds (heavy metals, toxins, etc), posing disposal challenges

applied in construction (Kostrzewa et al., 2023; Górka et al., 2023). Thus, the application of recovered sand from WWTP will be possible to enhance the efficiency of AD process while promoting circular economy practices and reducing the overall environmental footprint. Nonetheless, given the variability in sand characteristics across facilities, including differences in residual organics, heavy metals, and particle size, its application in full-scale systems requires careful consideration. In this study, we opted to use sand from a beach (previously washed, autoclaved, and calcinated) to guarantee results reproducibility. Nevertheless, to ensure safe and effective implementation, we recommend that sand from WWTPs undergo basic quality screening and characterization before use. Establishing such minimum quality criteria is essential for replicability, safety, and overall process stability in practical applications (Judd et al., 2017).

While sand showed clear short-term benefits in batch assays, its long-term performance in continuous anaerobic systems remains to be evaluated. Sand particles may stratify or become embedded within the biomass, potentially diminishing their physical effect or causing reactor clogging (Karthikeyan and Visvanathan, 2013). Sand might also present some abrasive effect, which can lead to increased wear on mechanical components (stirrers, pumps) of AD reactors (Rocamora et al. 2020; Karthikeyan and Visvanathan, 2013). This might result in higher maintenance needs and shorter equipment lifespan. Although we do not foresee a continuous addition of sand to AD reactors, the continuous addition could increase total solids loading, leading to the accumulation of solids at the bottom of reactors, reducing the working volume of digesters.

To strengthen the environmental and economic relevance of these findings, future studies should include detailed life cycle assessment and techno-economic analysis to quantitatively validate the sustainability benefits of sand, especially when sourced from existing wastewater infrastructure, where its reuse could offset material and disposal costs.

5. Conclusions

This study highlights the significant potential of sand, a non-CM, in enhancing biomethane production during AD. Our findings emphasize the advantages of using low-cost, environmentally friendly materials like sand to improve AD efficiency. By demonstrating that sand can present similar results to traditional CM in specific contexts, this research challenges conventional views on the necessity of CM for optimizing methane production.

The results show that sand supports microbial growth and enhances methanogenesis, suggesting that its physical properties provide a favorable environment for microbial activity. This highlights the importance of understanding how non-CM, like sand, interacts with microbial communities in AD systems. For that, follow-up studies focusing on the microbiological and physicochemical interactions involved are necessary to elucidate the underlying mechanisms.

Furthermore, our study underscores the need for continued research into material effects on microbial dynamics within AD processes. By identifying sand as a viable non-CM alternative, this research contributes to developing sustainable waste management practices, supports renewable energy solutions with low environmental impact and low-cost materials, and promotes a circular economy and sustainability.

Ethical approval

This article does not contain any studies with human participants or animals performed by any authors.

CRediT authorship contribution statement

Nicolás Hoffmann: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cátia S.N. Braga:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Olga Rubilar:** Supervision, Visualization, Writing – review & editing. **Gustavo Ciudad:** Visualization, Writing – review & editing. **Gonzalo Tortella:** Visualization, Writing – review & editing. **Edward Hermosilla:** Conceptualization, Investigation, Methodology, Writing – review & editing. **M.Salomé Duarte:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Luciana Pereira:** Writing – review & editing, Conceptualization. **Andreia F. Salvador:** Writing – review & editing, Conceptualization. **Gilberto Martins:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.133163.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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