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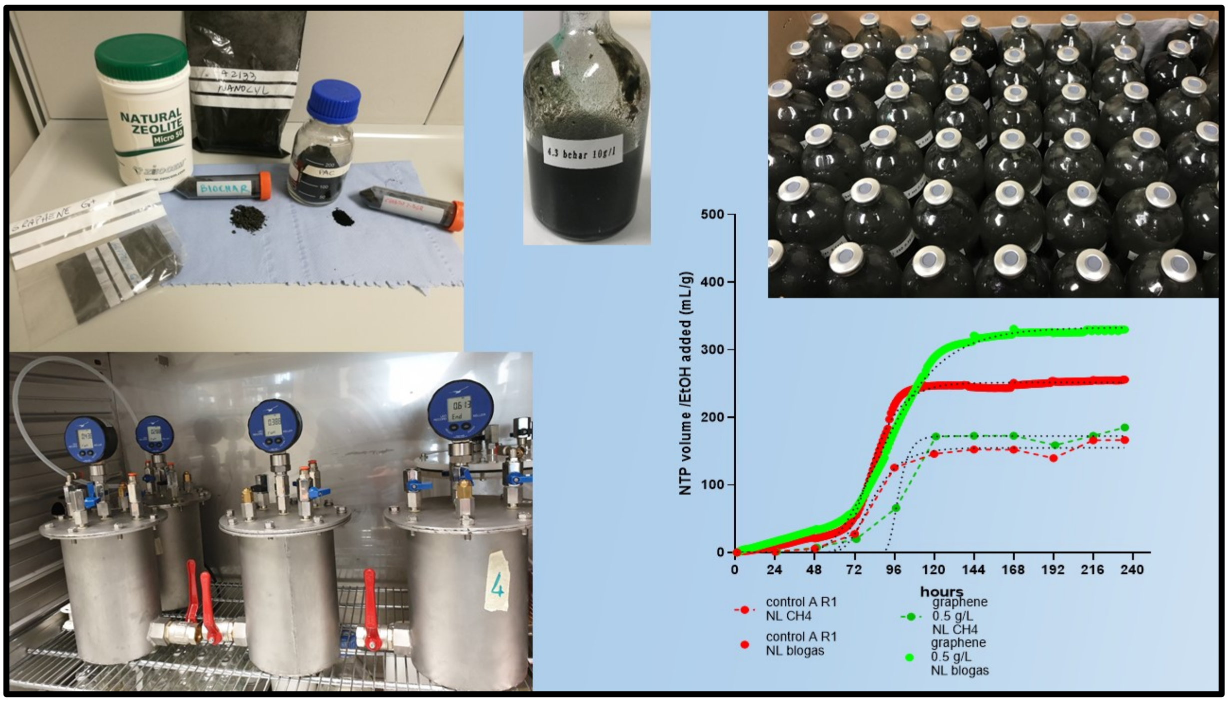
Final report dated 10 July 2019

Direct Interspecies Electron Transfer (DIET) to increase methane production



Source: BETlab SUPSI, 2019

University of Applied Sciences and Arts
of Southern Switzerland

SUPSI

Date: 10 July 2019

Location: Bern

Subsidiser:
Swiss Federal Office of Energy SFOE
Energy Research and Cleantech Section
CH-3003 Bern
www.bfe.admin.ch

Subsidy recipients:
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SFOE contract number: SI/501716-01

Zusammenfassung

Mittels anaerober Faulung werden Polysaccharide, Proteine, Nukleinsäuren und Lipide zu Wasserstoff, Formiat, Acetat und Kohlendioxid fermentiert und anschließend in Methan umgewandelt. Traditionell sind dabei vier unabhängige Schritte involviert: Hydrolyse, Acidogenese, Acetogenese, Methanogenese; Verschiedene Mikroorganismen interagieren miteinander, um komplexe organische Substanzen als Kohlenstoff- und Energiequelle zu verwerten wobei diese verschiedene Stoffwechselwegen einschlagen. Um die verminderte Stoffwechseleffizienz anaerober Prozesse zu überwinden, kooperieren die an der anaeroben Faulung beteiligten Mikroorganismen in einer als Syntrophie bezeichneten wechselseitigen Beziehung. Dieses Zusammenarbeiten der Mikroorganismen wurde unteranderem bei anaeroben Gärprozessen beobachtet. Syntrophische Mikroorganismen können, mithilfe von Trägersubstanzen oder über direkten Austausch in Form von Elektronen, chemische Energie übertragen. Im letzteren Fall ist der Prozess als Direct Interspecies Electron Transfer (DIET) definiert. Neuere wissenschaftliche Arbeiten versprechen sich durch die Verwendung von leitfähigen Materialien den Elektronenaustausch zwischen den Mikroorgansimen zu vereinfachen bzw. den Prozess positiv zu Unterstützen. Die synthrophischen Mikroorganismen die sich vom leitfähigen Material einen Vorteil verschaffen haben daher einen Wettbewerbsvorteil gegenüber den anderen Mikroorganismen. Dieser Vorteil führt schlussendlich zu einer höheren Biogas- und Methanausbeute. Die Zugabe von Graphen, Pflanzenkohle und Aktivkohle führten zu einer erhöhten Biogasproduktion im Vergleich zur jeweiligen Kontrolle. Das einzige getestete Material welches zu einer verminderten Biogasproduktion führte war Zeolith. Da Zeolith das Material mit der tiefsten Leitfähigkeit (nicht leitend) ist, stimmt dieses Ergebnis mit der Hypothese eines Elektronentransfervorteils durch die Zugabe von leitfähigen Materialien überein. Die Leitfähigkeit der Materialien ist abnehmend; Graphen, dann PAC, Pflanzenkohle und am Schluss Zeolith. Diese Abfolge entspricht auch der erreichten Steigerung des Biogasertrages.

Ergebnisse

- Um eine zuverlässige Aussage über den Effekt des Materiales zu treffen hatten die Versuche in 150 ml Reaktoren einen zu großen Messfehler.
- Die 3.5L-Reaktoren zeigten zuverlässige Ergebnisse in Bezug auf die kumulative Biogas- und Methanproduktion. Die Produktionsverläufe zeigen eine gute Übereinstimmung mit der Gompertz-Modellgleichung.
- Die Zugabe der unterschiedlichen Leitfähigen Materialien führte, unter den beschriebenen Testbedingungen, zu einer erhöhten Biogas- und Methanproduktion.
- Der Zusatz von Zeolith als nicht leitfähiges Material führte zu einem leichten Rückgang der kumulativen Biogasproduktion.
- Unter den getesteten leitfähigen Materialien (Graphen, PAC und Pflanzenkohle) bewegt sich die Biogaserhöhung parallele zur Steigerung der Materialleitfähigkeit. Zeolith (nicht leitend) reduzierte die Biogasproduktion.
- Es wurde kein Zusammenhang zwischen Materialkonzentration und Biogasproduktion festgestellt.

Riassunto

Tramite il processo di Digestione Anaerobica (DA), polisaccaridi, proteine, acidi nucleici e lipidi vengono fermentati in idrogeno, acido formico, acido acetico ed anidride carbonica, per poi essere convertiti in metano. Quattro passaggi separati sono tradizionalmente identificati nel processo: idrolisi, acidogenesi, acetogenesi, metanogenesi; diversi microrganismi interagiscono per sfruttare complesse matrici organiche come fonte di carbonio ed energia lungo diversi percorsi metabolici identificabili con i passaggi citati precedentemente. Per superare la ridotta efficienza metabolica dei percorsi energetici anaerobici, i microrganismi coinvolti nella digestione anaerobica cooperano in un mutuo rapporto denominato sintrofia. Questo fenomeno è stato osservato nei processi di digestione anaerobica. Microrganismi sintrofici possono trasferire energia chimica sotto forma di composti solubili, tramite carriers o per scambio diretto di elettroni. In quest'ultimo caso, il processo è definito Direct Interspecies Electron Transfer (DIET). Articoli scientifici recenti indicano la possibilità di sfruttare il processo DIET utilizzando materiali conduttivi che dovrebbero facilitare lo scambio di elettroni. I microrganismi sintrofici che beneficiano della presenza di materiale conduttivo hanno pertanto un vantaggio competitivo rispetto ad altri microrganismi, portando a rese di biogas e metano più elevate. L'aggiunta di grafene, biochar e carbone attivo ha mostrato una produzione di biogas maggiore rispetto al loro controllo. L'unico materiale aggiunto che ha mostrato un decremento della produzione di biogas è la zeolite. Essendo la zeolite l'unico materiale testato non conduttivo, questo risultato è in linea con l'ipotesi di un miglioramento dovuto al trasferimento elettronico. I valori di conduttività misurati mostrano il grafene più in alto, poi carbone attivo, biochar e infine zeolite: la stessa sequenza è stata osservata riguardo gli incrementi della produzione di biogas.

Risultati principali

- I test effettuati nei reattori da 120 mL hanno mostrato un errore troppo elevato per poter ottenere informazioni significative.
- I test effettuati nei reattori da 3,5 L hanno mostrato risultati in termini di produzioni cumulative di biogas e metano che si adattano bene con il modello dell'equazione di Gompertz e sono pertanto considerati risultati affidabili.
- L'aggiunta di materiali conduttivi in condizioni sperimentali nel processo di digestione anaerobica ha mostrato una produzione maggiore di biogas e di metano.
- L'aggiunta di zeolite come materiale non conduttivo ha mostrato una leggera diminuzione nella produzione cumulativa di biogas.
- Fra i diversi materiali conduttivi testati (grafene, carbone attivo e biochar), la sequenza di aumento di produzione di biogas segue la sequenza dei valori della scala delle conduttività dei materiali testati. La zeolite (non conduttivo) ha diminuito la produzione di biogas.
- Non è stata trovata alcuna relazione fra la concentrazione dei materiali testati e la produzione di biogas.

Summary

By means of Anaerobic Process (AD) polysaccharides, proteins, nucleic acids, and lipids are primarily fermented to hydrogen, formate, acetate, and carbon dioxide, and converted subsequently in methane. Four separate steps are traditionally identified in the process: hydrolysis, acidogenesis, acetogenesis, methanogenesis; different microorganisms interact to exploit complex organic matter as a source of carbon and energy in diverse metabolic pathways that are identified with the four steps above cited. To overcome the reduced metabolic efficiency of anaerobic pathways, the microorganisms involved in anaerobic digestion cooperate in a mutualistic relationship called syntrophy. This occurrence has been observed in Anaerobic Digestion processes. Syntrophic microorganisms can transfer chemical energy in the form of soluble chemical compounds, by carriers or by direct exchange of electrons. In this case, the process is defined Direct Interspecies Electron Transfer (DIET). Recent scientific papers suggest the possibility to exploit the DIET process by using conductive materials that are supposed to facilitate the electron exchange. The synthrophic microorganisms benefiting from the conductive material presence have therefore a competitive edge against the other microorganisms resulting in higher biogas and methane yields. Graphene, biochar and activated carbon resulted in an increased biogas production compared to their respective control. The only material addition that resulted in a decrease in the biogas production is zeolite. As zeolite is the only material tested with no conductivity, this result is in agreement with the hypothesis of an electron transfer benefit. The conductivity-scale values have graphene, then PAC, biochar and last zeolite: the same sequence is observed for the increase biogas production.

Main findings

- The tests carried out at 120 mL capacity reactors showed a too high error to obtain meaningful information.
- The tests carried out at 3.5 L reactor gave results in terms of cumulative biogas and methane production that fitted well with gompertz model equation and are therefore considered reliable.
- The addition of conductive materials to anaerobic digestion process in the test conditions resulted in higher biogas and methane production.
- The addition of zeolite as non-conductive material resulted in a slight decrease in the cumulative biogas production.
- Among the different conductive materials tested (graphene, PAC, and biochar) the sequence of biogas increase reflects the conductivity-scale values. Zeolite (non-conductive) reduced the biogas production.
- No relationship has been found among material concentration and biogas production.

Content

Abbreviations

AD: Anaerobic digestion

ATP: Adenosin Tri Phosphate

CNT: Carbon Nanotubes

DIET: Direct Interspecies Electron Transfer

DoE: Design of Experiment

MES: microbial electrosynthesis

MFC: Microbial fuel cell

NTP: Normal Temperature and Pressure values (15°C, 1 bar)

σ: Electrical conductivity A2s3/m3Kg

1 Introduction

1.1 Background information and current situation

Anaerobic digestion (AD) is one of the possible **processes to convert biomasses** and waste biomasses **into** added-value products such as **biogas**.

The AD process to degrade chemical compounds **is a multi-step process**, in which polysaccharides, proteins, nucleic acids, and lipids are primarily fermented to hydrogen, formate, acetate, CO_2 and converted subsequently in methane. The four steps are hydrolysis, acidogenesis, acetogenesis and methanogenesis.

The reaction involved in the last two steps of the AD process are thermodynamically linked: the butyrate and propionate degradation reactions are endergonic (Figure 1) and are therefore not favorable. To be able to gain energy from the butyrate and propionate degradations, these reactions have to be coupled with the thermodynamically favorable methanogenesis and the maximum amount of energy released is equivalent to about one ATP (Amani, Nosrati, and Sreekrishnan 2010).

	Biochemical reactions	$\Delta G°$ @25°C (kJ/mol)
acetogens	$butyrate^- + 2H_2O \rightarrow 2\,acetate^- + 2H_2 + H^+$	+48.1
	$butyrate^- + 2HCO_3^- \rightarrow 2\,acetate^- + 2formate^- + H^+$	+45.5
	$propionate^- + 3H_2O \rightarrow acetate^- + 2HCO_3^- + H^+ + 3H_2$	+76.1
	$propionate^- + 2HCO_3^- \rightarrow acetate^- + H^+ + 3formate^-$	+72.2
methanogens	$H_2 + {}^1/_4 H_2O + {}^1/_4 H^+ \rightarrow {}^1/_4 CH_4 + {}^3/_4 H_2O$	-33.9
	$formate^- + {}^1/_4 H_2O + {}^1/_4 H^+ \rightarrow {}^1/_4 CH_4 + {}^3/_4 HCO_3^-$	-32.6
	$acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0

Figure 1: biochemical reactions involved in the AD process modified from (Amani, Nosrati, and Sreekrishnan 2010)

It is known by literature that **different trophic groups of microorganisms cooperate in a syntrophy** to exploit complex organic matter as source of carbon and energy. Syntrophy has been defined as obligate mutualistic metabolism (Morris et al. 2013). Syntrophy has been shown to occur in anaerobic digestion processes. Obligate syntrophic communities consist of microorganisms with metabolisms that are thermodynamically linked and catabolically interdependent. (Embree et al. 2015). Syntrophy occurs by the transfer of chemical energy in the form of soluble chemical compounds between two organisms and between anoxic and methanogenic environments. Besides the exchange of diffusible molecules and energy carriers such as hydrogen or formate, microorganisms can transfer electrons in a more direct way such as via conductive pili (González-Fandos 2015).

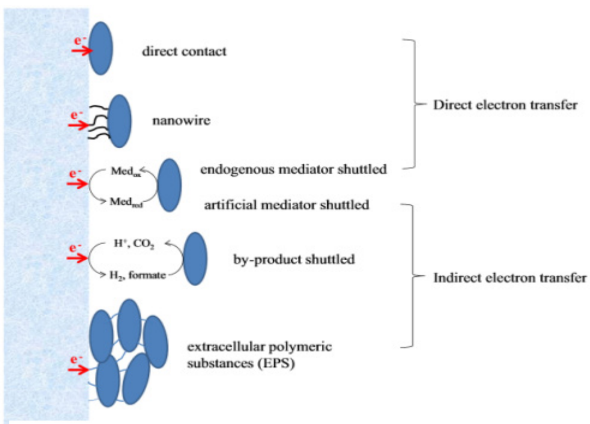


Figure 2: Extracellular electron transfer from Choi *et al.*, 2016.

Among the four different steps involved in the overall transformation from organic matter to methane, the last step carried out by the strictly anaerobic methanogens is the most sensitive as the methanogens are slow growing, have low resistance against environmental changes and can convert to methane just few molecules (Mayer and Mu 2014).

One of the most important critical point of the AD process is the long start-up time due to the microbial lag phase and the elapsed time in case of system failure to grow back the biomass.

Different bacteria (named "electroactive") have evolved strategies to transfer electrons far beyond the cell surface. According to the direction of electron flow, the microorganisms transfer electrons from organic compound (anode) to produce electricity as in Microbial fuel cell (MFC), or use external electron flow to synthetize organic compounds (cathode) as in microbial electrosynthesis (MES). The mode of extracellular electron transfer from cathode can occur by direct or indirect electron transfer; see Figure 2 (Choi and Sang 2016).

Direct interspecies electron transfer (DIET) has been proposed as syntrophy mechanism; two microbial species exchange electrons via electric currents flowing through conductive solid conduits such as microbial pili, but also conductive material (see Figure 3). With this process different microorganisms in a community are able to share reducing equivalents to drive the methanogenic degradation of organic substrates (Kouzuma, Kato, and Watanabe 2015).

Recent studies have suggested that **conductive iron oxide minerals can facilitate syntrophic metabolism of the methanogenic** degradation of organic matter such as ethanol, propionate and butyrate in natural and engineered microbial ecosystems.

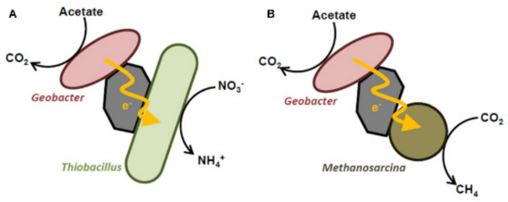


Figure 3: Schematic diagrams showing electric syntrophy between *Geobacter* spp. and *Thiobacillus denitrificans* (A) and *Methanosarcina* spp. (B) mediated by conductive material (Kouzuma, Kato, and Watanabe 2015).

Different conductive carbon materials have been proved able to support and facilitate DIET process in lab scale with pure culture microorganisms. They including graphite particles (Kato, Hashimoto, and Watanabe 2012), granular activated carbon (Liu *et al*. 2012), biochar (Chen, Rotaru, Shrestha, *et al*. 2014), and carbon cloth (Chen, Rotaru, Liu, *et al*. 2014).

Cruz Viggi *et al*. (2014) tested the addition of microparticulated magnetite (Fe_3O_4) in a real anaerobic digestion process. The authors report having triggered a Direct Interspecies Electron transfer DIET and have proposed a shortcut route of propionate conversion into methane that is faster and less sensitive on external H_2 partial pressure than the "classical" one based on interspecies H_2 transfer. They observed an increased methane formation rate of around 31−33%.

Many papers recently published research data on anaerobic digestion processes enhanced by mechanisms involved in the DIET process. However as also reported in (Park et al. 2018), there is still the need to relate the effect of the material addition with conductivity values and physical properties as the material characteristics (dimension, surface) may affect the DIET efficiency.

1.2 Purpose of the project

The project investigates the possibility to use conductive materials to maximize the methane production in anaerobic digestion processes. The idea is to exploit the DIET mechanisms by adding conductive materials in the digesters. A relationship between the material characteristics and the increment in the biogas production would be useful in optimizing the methane production.

1.3 Objectives

The project general objective is to test the suitability of five different materials to trigger the DIET mechanism resulting in an increased methane production.

Compare the kinetics of biomethane production with additions of different materials at different concentration, and relate the methane production to the conductive values and particle size.

2 Description of facility

The research is described in the procedure and methodology section.

3 Procedures and methodology

Selection of the material. Five materials known to have different conductive properties have been chosen: Graphene (Directaplus, Pure G+), Multiwall Carbon nanotubes (Nanocyl, NC7000), Activated carbon (Norit), Biochar (Verora), Carbon fiber (FC Carbon), Zeolite (Zeocem, micro50).

Material characterization. Conductivity values were not reported homogeneously in the material technical datasheet; for this reason the electrical conductivity (σ) has been defined for each material using the same approach: the electrical resistance (Ω) has been measured by compacting each material in a chamber and applying the same force, then the values have been converted in electrical conductivity (σ) by applying the formula (σ)= (A^2s^3/m^3Kg). In Figure is reported the device used to measure the electrical resistance of the materials.

The structure characterization has been obtained by measuring the particle size with SEM. For each material eight measures were recorded and mean value and standard deviation noted.

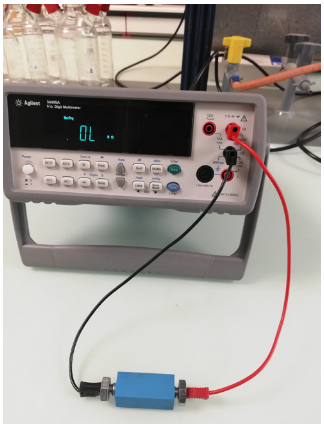


Figure 4: device developed by HM lab to measure electrical resistance.

Microscope observation. Materials samples were mounted on conductive tape and observed using a scanning electron microscope (SEM, InTouchScope JSM-6010LA, JEOL, Japan)with the filament operating at 10 KeV acceleration voltage and magnification up to 4500x.

Experimental design and data processing. Design Expert software (Stat-Ease, Minneapolis, USA) version 10, was used for the design of experiments approach. A Response surface I-optimal design was employed in which two variables, filler typology and concentration, were varied at six and two levels respectively. A linear design model was adopted to analyze the response, i.e. the average theoretical biogas production. A total of seventeen experiments were selected and executed, which comprise five replicated tests for statistical reasons.

Two sets of batch test experiments (BioMethane Potential) tests were performed: the first test for screening concentrations and materials were set on a small scale: 120 mL-vials were filled with 40mL inoculum sampled from

from the anaerobic digester of Chiasso wastewater treatment plant, 0.5mL of Ethanol 99% (VWR International GmbH, Dietikon) as sole carbon source and 19.5mL of water solution with the suspended conductive material. The concentration tested were chosen considering literature data tested range and selecting the lower and higher values ((Yang et al. 2017; Lin et al. 2017; Park et al. 2018): for Activated Carbon 0.5g/L and 20g/l, Biochar 0.5g/L, 10g/L, Zeolite 0.5g/L, 10g/L, carbon nanotubes 0.1g/L, 5g/L, carbon fibers 0.1g/L, 5g/L, graphene 0.5g/L, 2g/L in triplicate.

The second set of tests were run on the most promising materials and were set on a larger scale in 3.58L capacity reactors filled with 1170mL inoculum sampled from digestate from the wastewater treatment plant in Chiasso, 20.55mL of Ethanol 99% (VWR International GmbH, Dietikon, CH) as sole carbon source, and 600mL of water solution with the conductive material suspended. The concentration tested were as follows: Activated Carbon 20g/L, Biochar 10g/L, Zeolite 10g/L, graphene 0.5g/L.

Each sample was mixed for 3–5 min before AD to obtain a homogeneous mixture and was flushed with nitrogen gas for about 2 min to assure AD conditions before the batch experiments. All reactors were shaken manually for 1 min each day during AD process.

Analytical techniques. The total solids (TS) and volatile solids (VS) content were determined in accordance with APHA Standard Methods (1995).

The biogas production was measured by manometer measuring the overpressure daily on the small scale set and automatically every hour for the higher scale reactors. The pressure values were converted to Liter of biogas in normal conditions (15°C, 1 bar) applying the ideal gas law PV=nRT.

Methane concentration. The methane concentration in the biogas has been measured by IR through the Gas Analyser (Biogas5000 Geotech, Lauper Instruments AG, Murten, CH) and expressed as %.

GC analysis. Preparation of standard gas. A certified gas standard mixture containing methane (44%) and air (56%) was purchased from Pangas (Dagmersellen, Switzerland, Ecocyl® RSH PG1 1L). The preparation of standard gas and sample analysis was carried out according to Liu et al. [1] with minor modifications. Briefly, a volume of standard gas was introduced in a Restek 1 liter Altef bag (RT-22959) at 37°C; increasing volumes of gas were injected into the chromatographic system (20µl/50µl/100µl/150µl/200µl) corresponding to the following methane concentrations (8.8%/22%/44%/66%/88%). Each condition was repeated at least 3 times and average peak area was used for quantitative analysis. **Samples analysis.** 100 µl of headspace gas was taken directly from the sample reaction vials at 37°C and injected into the chromatograph; analyses were carried out in duplicate. **Instrumentation.** Quantitative determination of methane was performed on a Restek RTx-5MS fused silica 30m, 0.25 mm, 0.25 µm column (RT-12623) installed on an Agilent GC 6890N instrument equipped with a flame ionization detector (FID). The oven was programmed with an initial temperature of 40°C for 5 min, increasing at 40°C min^{-1} to 200°C and then held for 1 min. The injector and FID detector were set, respectively, at 200°C and 240°C. High purity helium was used for carrier gas at 1.0 ml/min. The split ratio of gas sample in the inlet chamber was 20:1; hydrogen flow was set at 40 ml/min, air flow at 450 ml/min and make up gas (nitrogen) at 32 ml/min. Topaz inert liners (RT-23301) were used for injection.

Analysis of biogas production. Biogas production was analysed using a modified Gompertz equation (Lay, Li, and Noike 1997), which can estimate ultimate biogas volume, maximum biogas production rate, and lag time based on the following equation.

$$Y = Ym * \exp\left(-\exp\left(Rm * \frac{2.7186}{Ym} * (l - x) + 1\right)\right)$$

where Y (t) is the accumulative biogas production ($mLg^{-1}VS$) at an anaerobic digestion time t (d), Ym is the biogas production potential (mLg^{-1}-VS), Rm is the maximum biogas production rate ($mLg^{-1}VSd^{-1}$), l is the duration of lag-phase time (d), and e = 2.7183. Kinetic parameters were obtained by nonlinear regression fitting using GraphPad Prism (version 8.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) and least square regression method, goodness of fit quantified by R-squared.

4 Results and discussion

Material characterization. Scanning electron microscopy has been applied to study particle size distribution and morphology. The observation evidenced a large distribution of particle size, a wide morphology and the existence of a fractal distribution: zooming in new aggregates can be observed. Considering that the DIET mechanism is based on the interaction between a cell and the material, we decided to focus on the 1-10µm range to study the particle size distribution. For the same reason we decided to measure the conductivity of the material itself instead of a solution at different concentration.

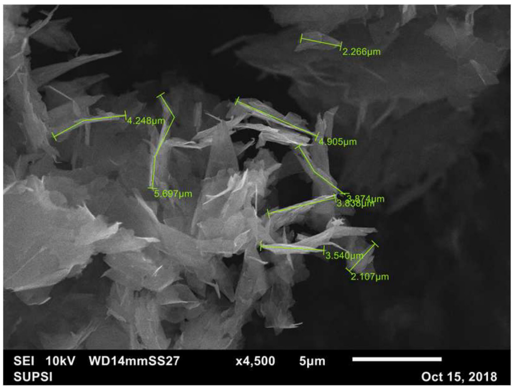


Figure 5: particle size measurement for graphene.

In Figure 5 are reported -as an example-, the measurements done for graphene samples.
Graphene is the material with the smallest particle size and also the more homogeneous distribution. It is followed by activated carbon and zeolite. Carbon nanotubes have the largest particle size (> 100µm).

Conductivity values. The materials selected to run the experiments are conductive powders available on the market, however their technical data sheets do not provide an actual value for the conductivity. To overcome this limitation measures of conductivity have been made on a small specimen of compressed powders. The system described in the material and methods section has been developed in HM lab in order to compress the powders and measures the conductivity.
An average value of conductivity as a function of the powder density has been measured and the data are reported in Table 1. Zeolite data are not shown in the table as the material is non-conductive.

Table 1: conductivity values for the materials used in the experiments

CNT		Biochar		Graphene		Act carbon		carbon fiber		
d g/cm³	σ S/m	d g/cm³	σ S/m	d g/cm³	σ S/m	d g/cm³	σ S/m	d g/cm³	σ S/m	
0.189	327.74	0.523	0.0023	1.216	432.27	0.465	2.65	0.769	0.0077	
0.224	381.65	0.533	0.0033	1.337	428.70	0.481	4.41	0.795	0.0265	
0.224	395.91	0.535	0.0038	1.349	425.13	0.491	5.05	0.809	0.0431	
0.244	441.20	0.543	0.0042	1.349	467.64	0.495	5.53	0.823	0.0636	
0.264	466.47	0.544	0.0045	1.372	525.46	0.503	6.07	0.832	0.1045	
0.282	454.58	0.549	0.0049	1.372	557.31	0.503	7.03	0.847	0.1788	
0.291	464.83	0.552	0.0051	1.384	569.81	0.528	10.31	0.868	0.3519	
0.316	453.71	0.554	0.0055	1.384	588.20	0.540	16.20	0.898	0.8592	
0.329	476.77	0.558	0.0058	1.384	607.80	0.549	18.68	0.909	1.0569	
0.344	466.24	0.564	0.0064	1.396	645.60	0.553	20.32	0.923	1.4302	
0.350	487.15	0.569	0.0069	1.396	695.26	0.564	23.16	0.926	1.8322	
0.353	493.20	0.571	0.0075	1.396	695.26	0.569	23.88	0.930	2.1845	
0.355	513.89	0.574	0.0080	1.396	753.20	0.570	25.52	0.946	2.8019	
0.357	520.88	0.574	0.0084	1.408	746.65	0.572	28.39	0.953	3.5509	
0.294	**453.158**	**0.553**	**0.0055**	**1.367**	**581.308**	**0.527**	**14.085**	**0.873**	**1.035**	Average

Biogas composition: GC analysis. The method to measure methane concentration has been set up with a 5 point-calibration curve to establish method linearity (R^2=0.99).

First screening BMP tests: the results obtained with the first set of tests (120mL capacity reactors) are reported in the Figure 6. In each figure it is reported the biogas cumulative production for each material at the different concentrations tested. The three replicates are reported as mean and standard deviation. The concentration tested is noted in the caption under the curves. The kinetic parameters obtained from non-linear fitting are reported in the Table 2.

Table 2: kinetic parameters obtained fitting with the mod.Gompertz equation.

	control	activated carbon PAC			biochar	zeolite			Carbon nanotubes CNTs		carbon fiber		graphene	
Conc.		0.5 g/L	20 g/L	0.5 g/L	0.5 g/L	0.5 g/L	10 g/L	10 g/L	0.1 g/L	5 g/L	0.1 g/L	5 g/L	0.5 g/L	2 g/L
Measured biogas production (mL/g EtOH)	375.67	719.18	652.41	530.03	659.87	929.03	957.06	853.37	692.30	969.06	1010.86	843.03	871.29	1006.50
Biogas production potential (mL)	341.94	704.97	686.91	518.71	641.17	914.43	939.87	820.97	673.47	977.97	991.28	807.26	844.57	987.46
Biogas production rate (mL/d)	0.13	0.11	0.07	0.07	0.10	0.14	0.14	0.14	0.11	0.10	0.10	0.13	0.13	0.14
Lag period (days)	2.90	1.82	1.52	1.87	1.78	2.03	1.71	1.46	1.73	0.73	1.66	1.16	1.72	1.30

Observing the cumulative curves reported in Figure 6, it is possible to note a high variance in the three replicates: for example activated carbon, CNT and biochar had high error. Also, a different behavior is obtained for the same concentration run twice. This high variability is not observed for graphene and zeolite. One possible explanation of the high error measured is the difficult dosing of powdered materials that do not dissolve: the measured small volumes uptaken for setting the tests had a large variation in the suspended material.

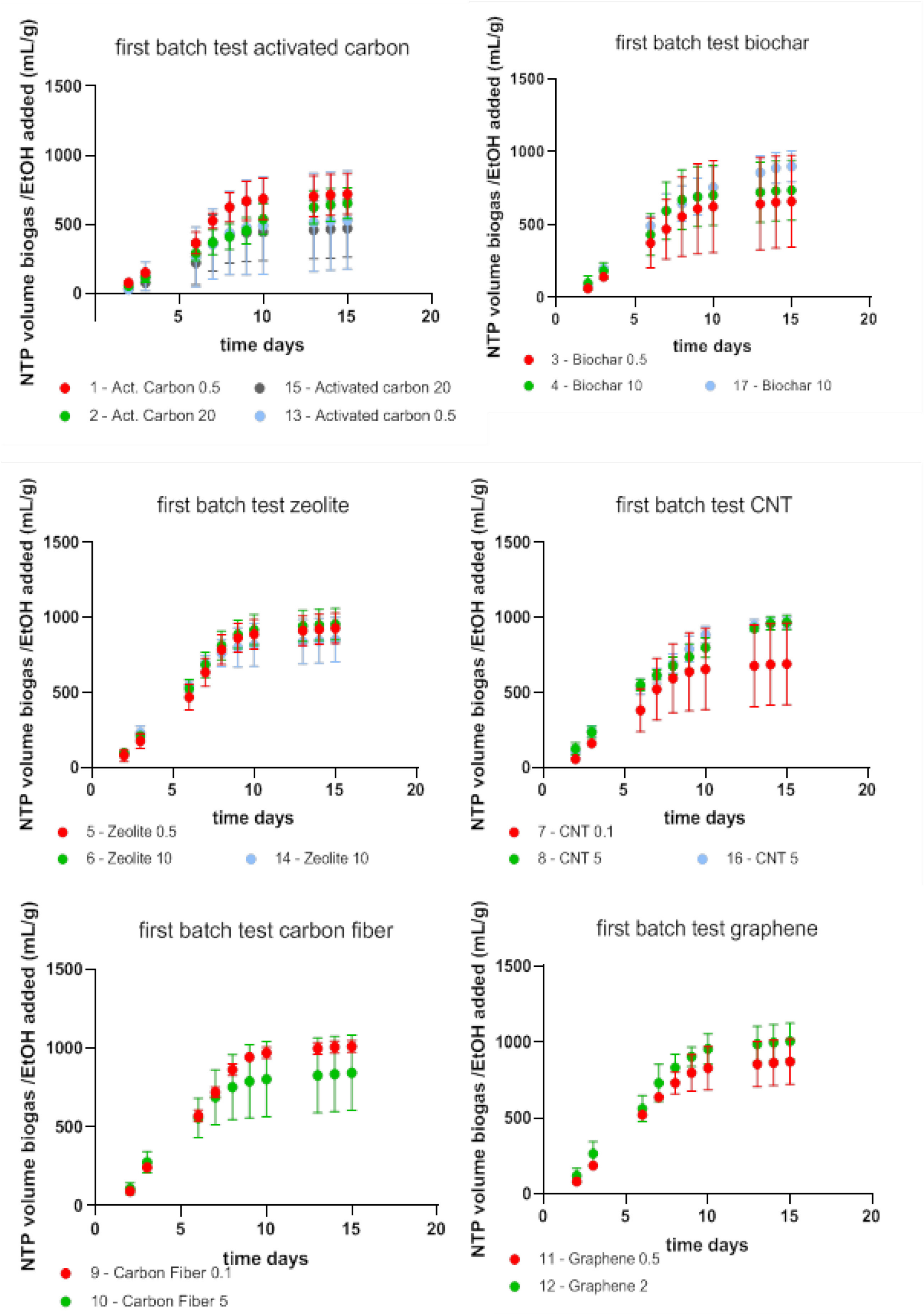


Figure 6: batch anaerobic digestion performance at various materials addition (n=3). The captions indicate the material concentration.

Despite the variability observed, the data were analysed by regression in order to identify the concentrations and materials significant by DoE; the parameter used in the DoE analysis is the max biogas potential production.

The results of the analysis is reported in Figure 7: Zeolite, CNT, Carbon fiber and Graphene determine the highest average theoretical production of biogas and they group together as a single entity. Their concentration is not significant, considering that, this factor can be excluded from the experimental plan analysis.

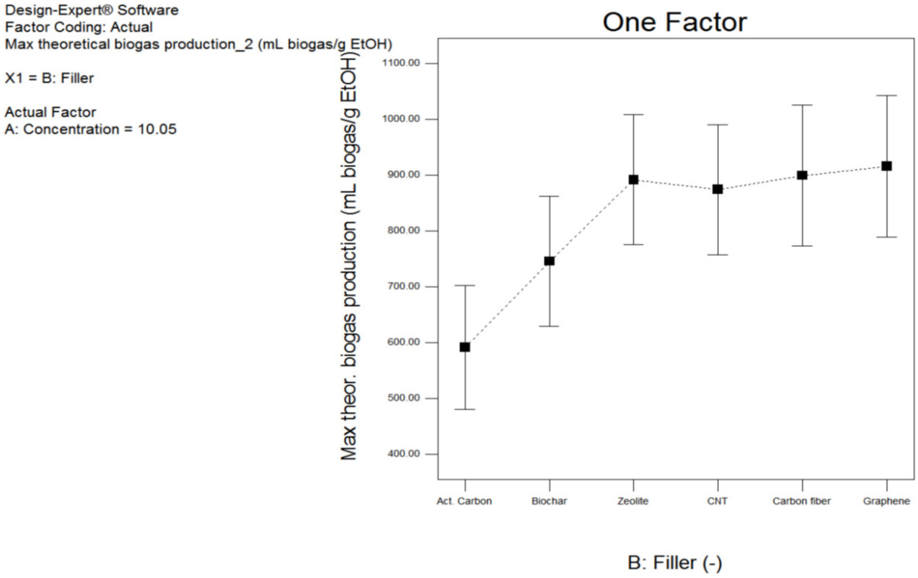


Figure 7: grouping of the materials (filler) tested by max theoretical biogas production.

The analysis was run a second time considering the four best performing materials as single unit (zeolite, CNT Carbon fiber and graphene). The result is reported in Figure 8 and it showed no significant difference within the materials. The filler Activated carbon gave the lowest production amount of biogas whereas the Biochar exhibited an intermediate value. <u>The concentration in the test condition, turned out to be not significant, considering the range of values adopted for each factor</u>.

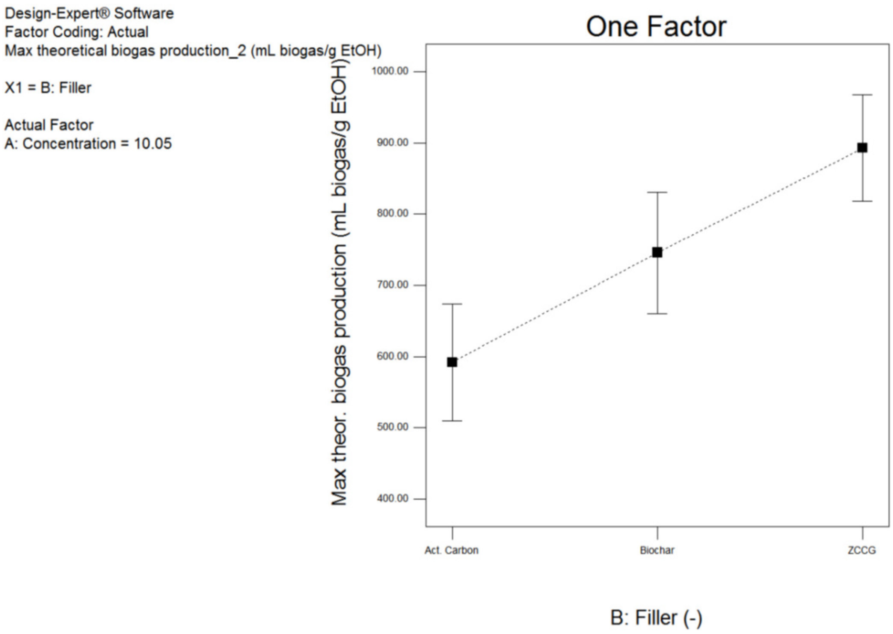


Figure 8: relationship between the best performing materials and the other tested.

Effect of the material in the biogas production. In order to evaluate the effect of the material addition the cumulative curves of biogas production were compared with the control run with the same experimental settings except for the presence of the material. The tests were run at the same time for all the material in triplicate. Data are shown as mean of three replicates with shaded the standard deviation. In the Figure 9 are reported the results obtained for zeolite comparing each concentration tested with the corresponding control.

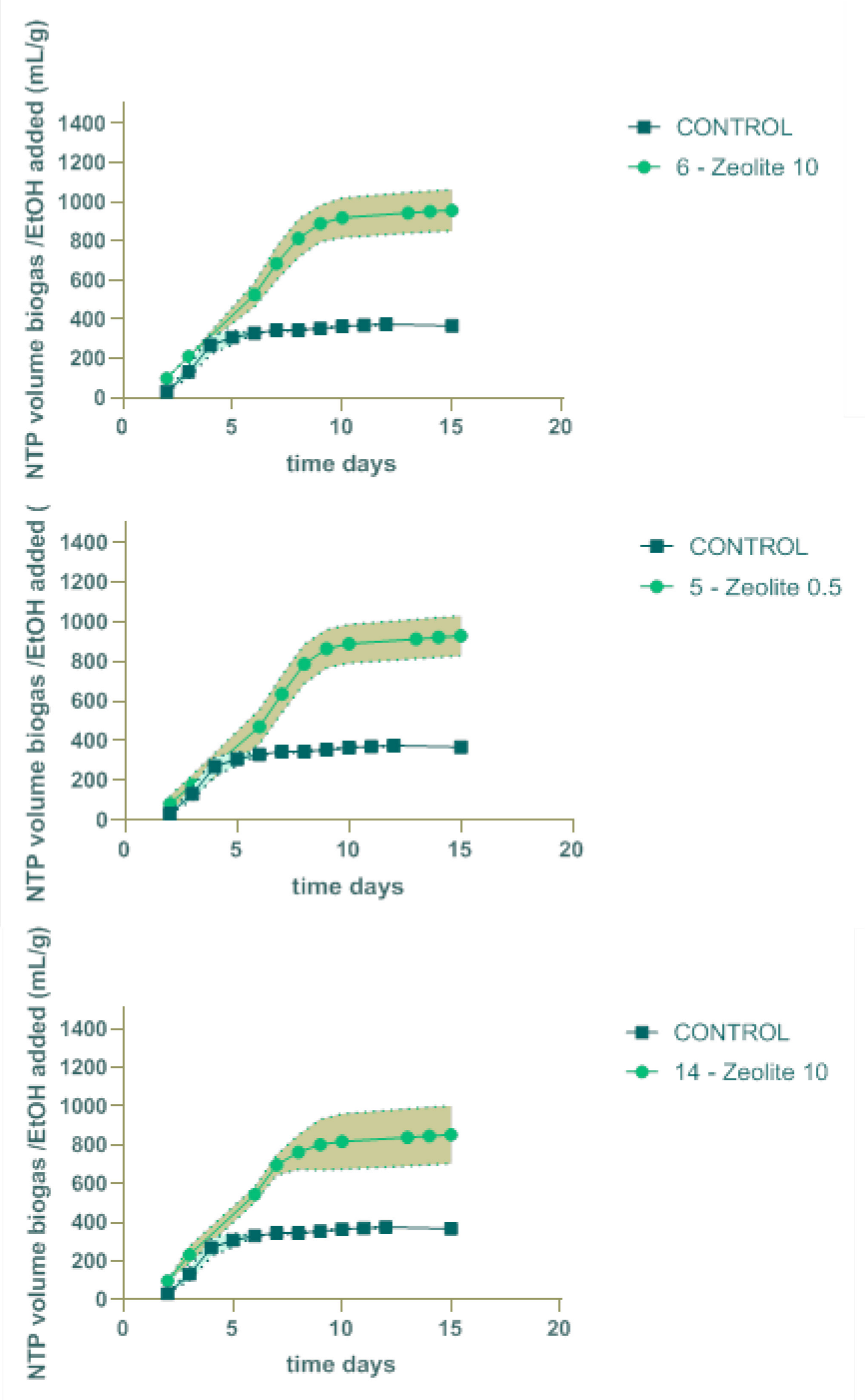


Figure 9: BMP tests for zeolite addition at different concentrations compared to the control

The tests run with zeolite addition showed a difference in the trend of the cumulative biogas production starting from day 6 forward. The cumulative production at the end of the experiments (15 days) were higher than the control for all the concentration tested.

In the Figure 10 the results obtained for CNT Carbon Nanotubes are reported. The CNT at 0.1 g/L showed a high variability in the three replicates, with an overall higher biogas production compared to the control.

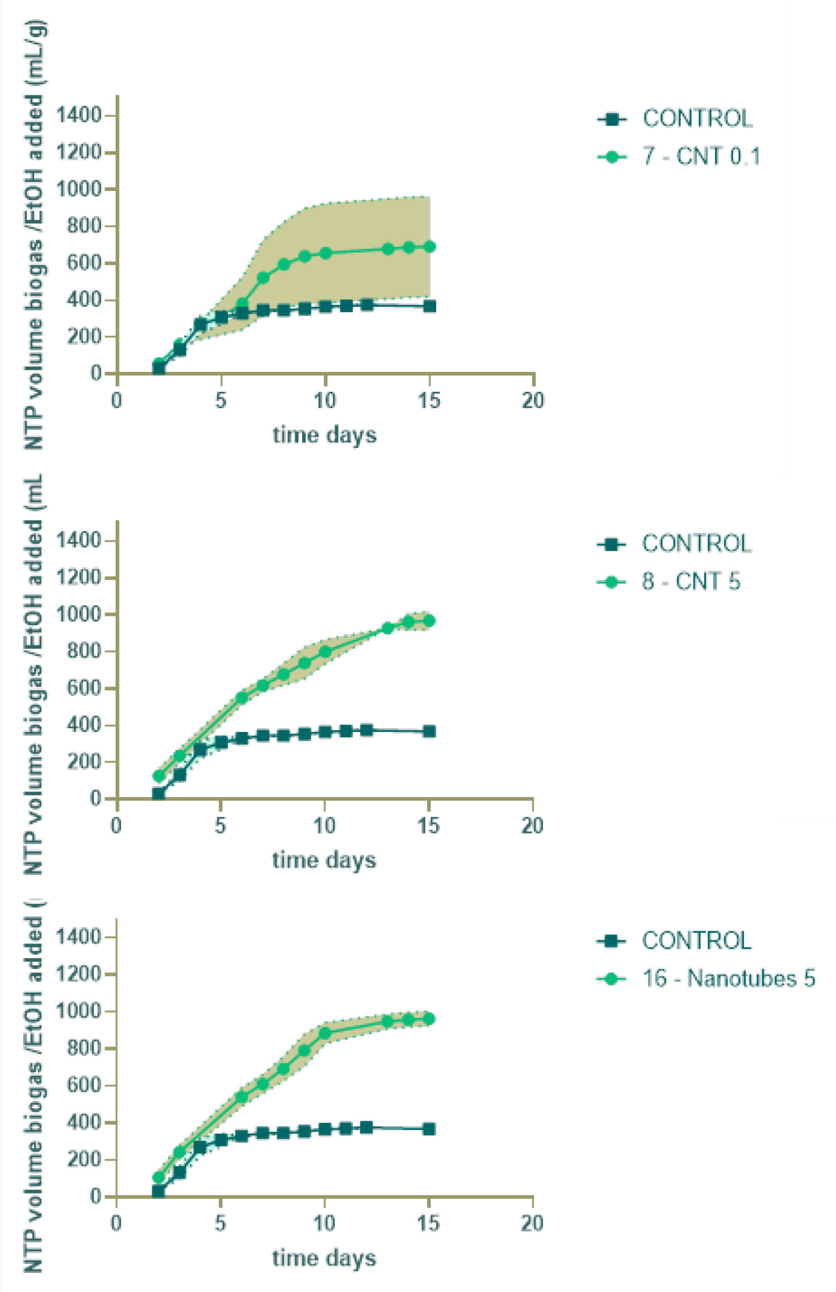


Figure 10: BMP tests for carbon nanotubes addition at different concentrations compared to the control.

In the Figure 11 the results obtained for Carbon Fibers are reported. The test run at 5 g/L showed a high variability in the three replicates, with an overall higher biogas production than the control.

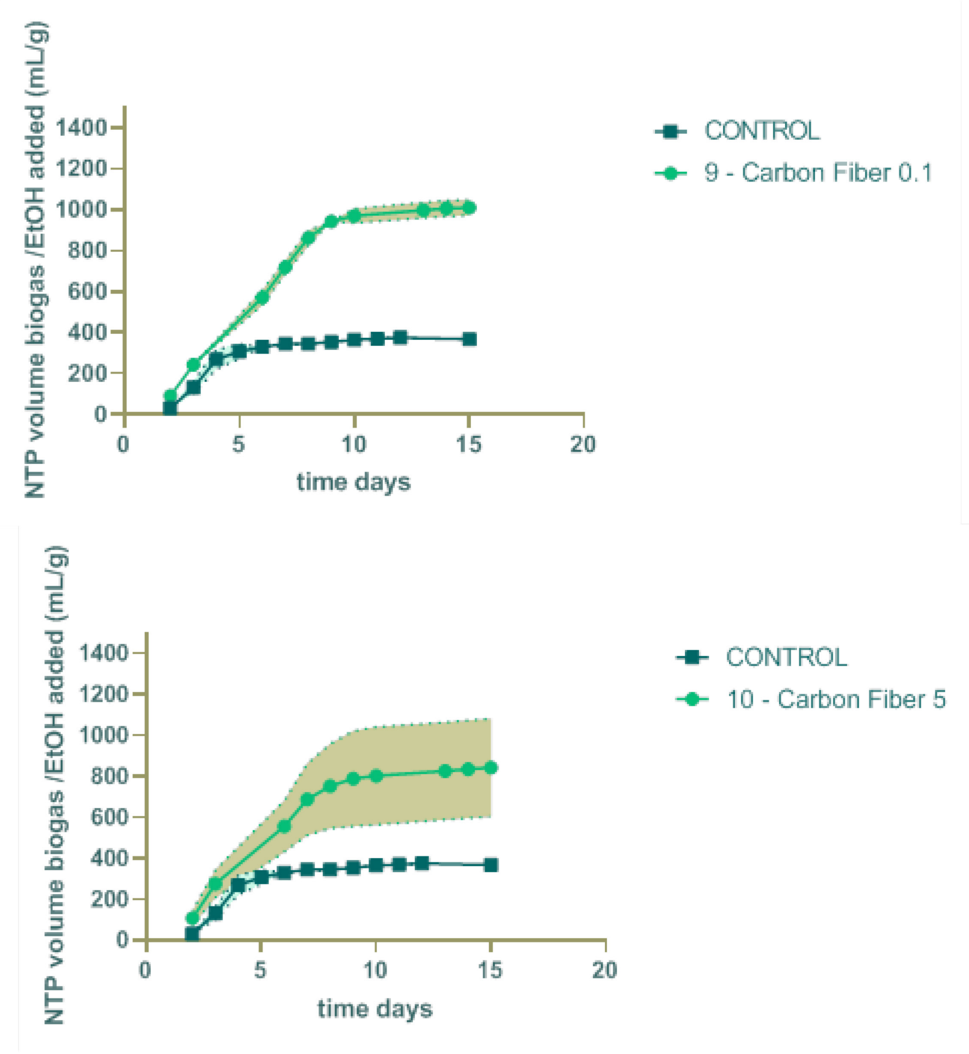
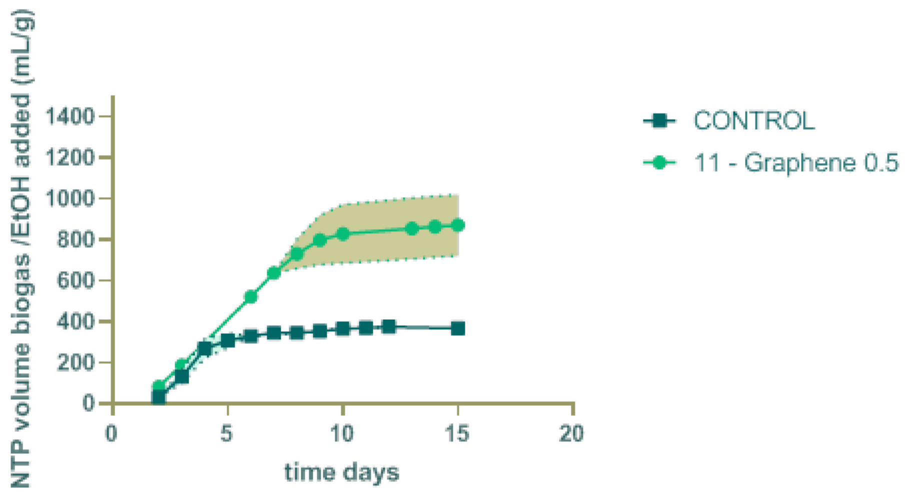


Figure 11: BMP tests for carbon fiber addition at different concentrations compared to the control.

The results obtained for graphene addition are reported in Figure 12. Also in this case, the tests run with the addition of material had a different curve trend with a higher biogas production.



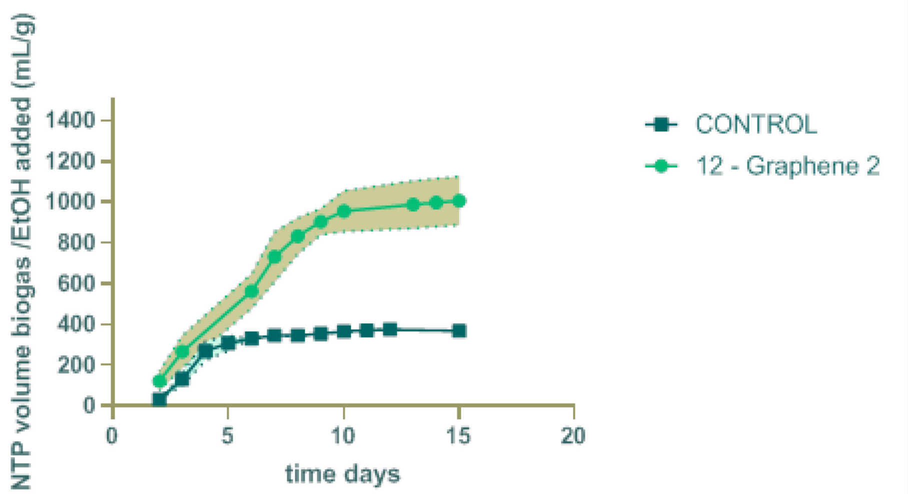


Figure 12: BMP tests for graphene addition at different concentrations compared to the control.

The data obtained for activated carbon addition are shown in Figure 13 and 14. Two concentration were tested (0.5 and 20 g/L), and the experiments were set twice. While the first two tests (graph on the left) showed a close variability enough to evidence a difference with the control, the second couple of tests (graphs on the right) had higher errors.

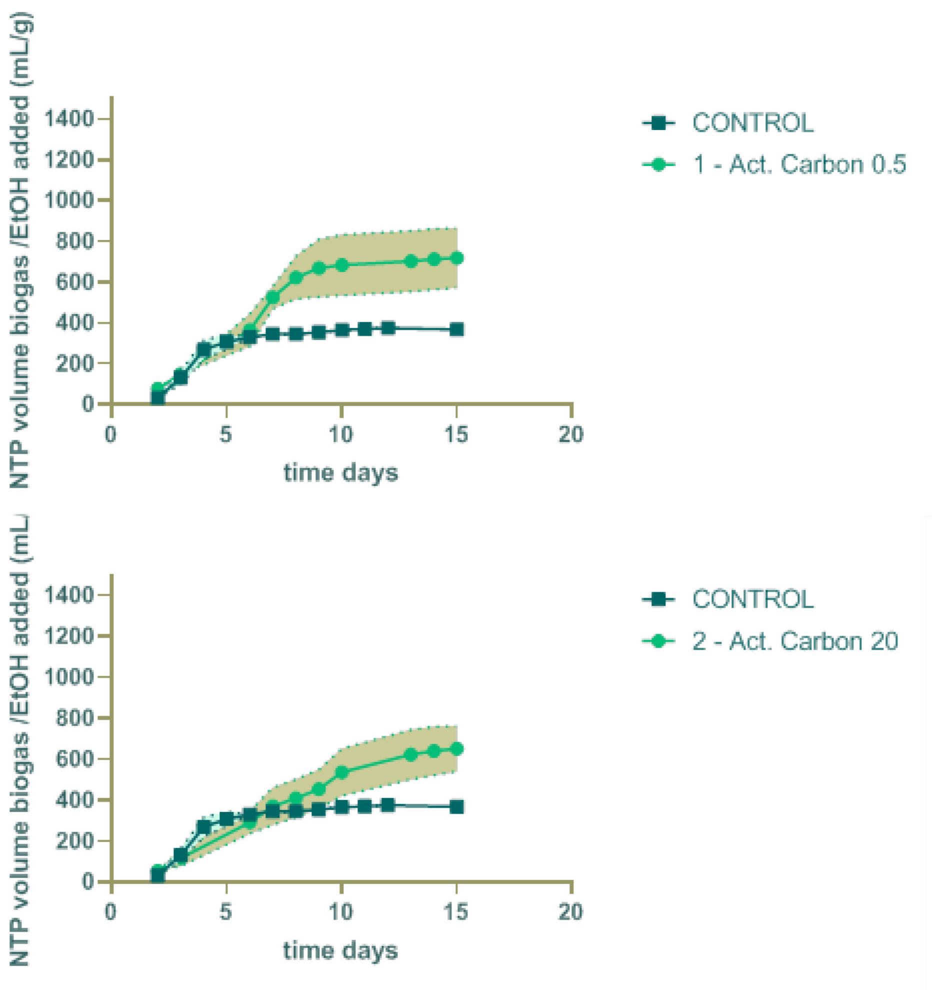


Figure 13: BMP tests for activated carbon addition at different concentrations compared to the control, first run.

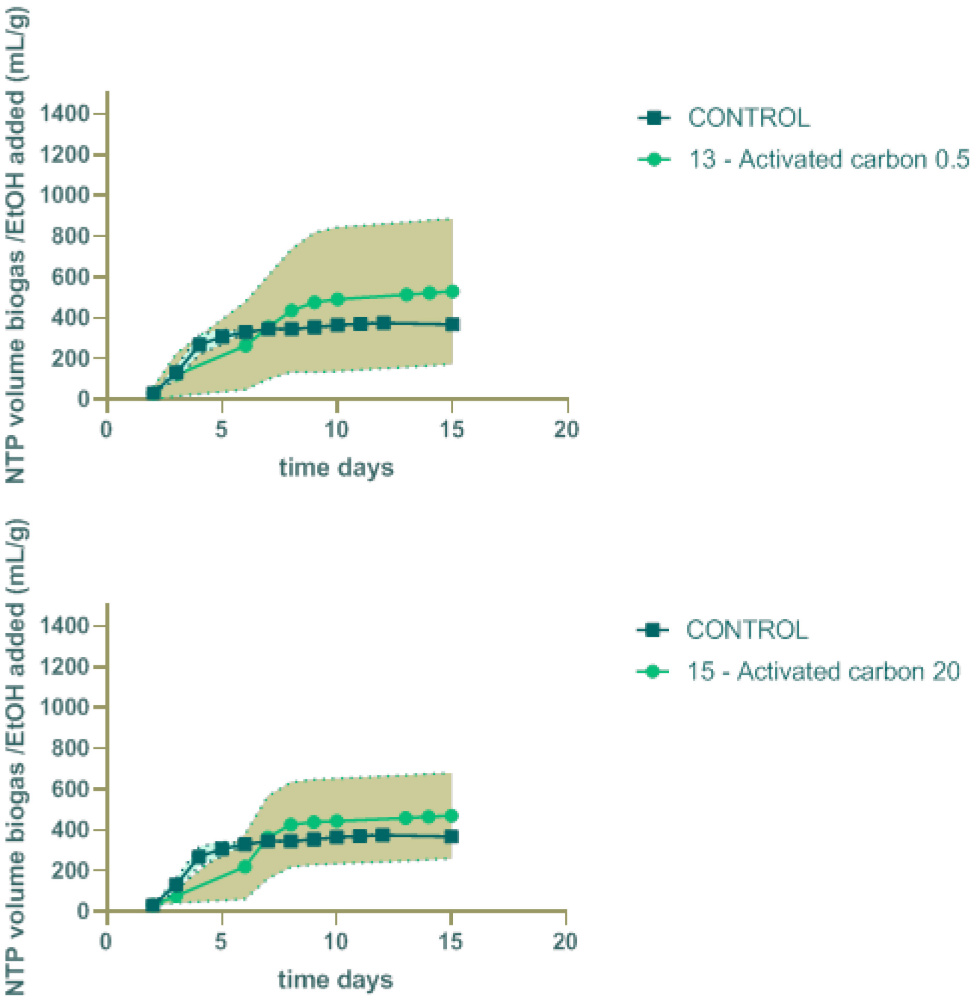


Figure 14: BMP tests for activated carbon addition at different concentrations compared to the control, second run.

The same issue has been observed for biochar addition (reported in Figure 15). The tests set at 10 g/L were duplicated and the results were not comparable.

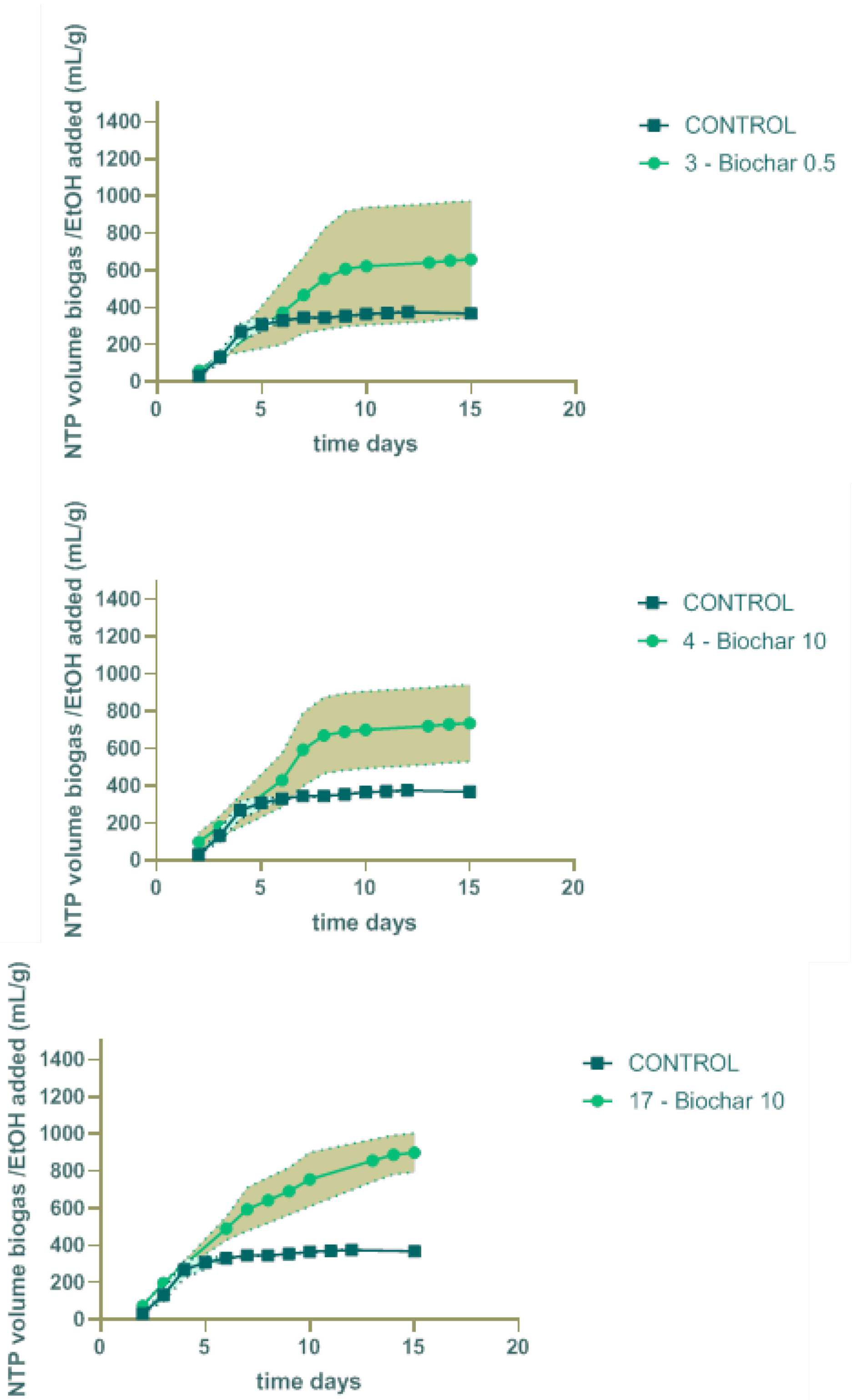


Figure 15: BMP tests for biochar addition at different concentrations compared to the control.

Methane concentration in biogas. From each vial for the tests Control, Zeolite, Carbon Nanotubes, carbon Fibers and Graphene at 2, 6, 8 and 10 days of the process, biogas samples were collected in vials and shipped to the "Fondazione Alpina" for GC measurements. Due to the small volume produced daily, biogas composition by IR measurements was not feasible. The results obtained are reported in Table 3.

Table 3: Methane percentage values measured in the biogas by GC analysis.

Material concentration g/L		% CH_4			
		Process days			
		2	6	8	10
Control	None	33.08	67.64	63.6	64.35
Zeolite	0.5	34.61	64.63	64.23	62.75
Carbon Nanotubes	0.1	34.87	65.37	65.49	62.87
Carbon Fibers	0.1	36.4	63.99	65.21	64.52
Graphene	0.5	35.44	64.74	62.76	62.99

Considering the results obtained in the whole set of "small scale tests" it is possible to observe that the addition of material to the tests caused an increase in biogas production. The data collected at the steady state that is from day 10 to the end, have been used to evidence differences among the tests. The one way ANOVA results carried out with Tukey's post Hoc tests (GraphPad Prism, version 8.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com), are reported in Figure 16. The cumulative mean biogas production is reported in crescent order; the control showed a lower biogas production and it is different from the value obtained in all the other tests. The materials group together with no effect of concentration or conductivity. In fact, among the highest biogas production there are the tests with the non-conductive zeolite.

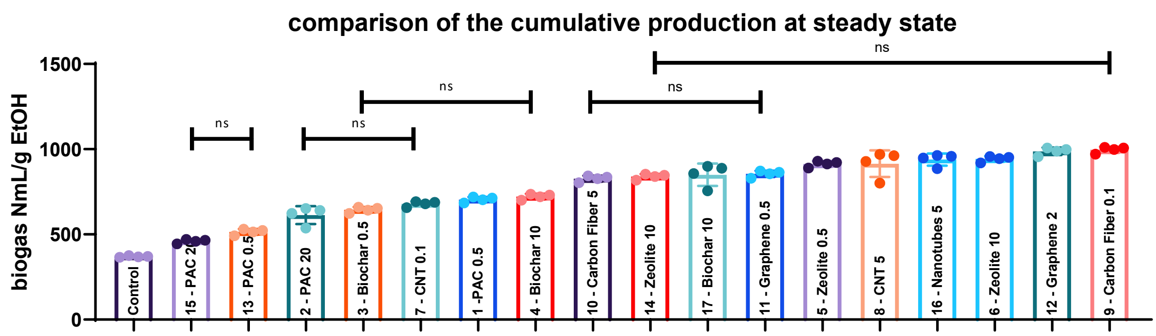


Figure 16: cumulative biogas production means on data collected during the steady state (days 5 to 10) for each test. The lines above the histograms group tests that resulted non different at α 0.05 significance value.

Considering the data obtained for all the tests, it can be concluded that the error in the replicates was an important issue: in fact, the error even if large was not constant. The problem is probably related to the small volume setting of the tests and to the difficulty in dispensing the weighted materials. This last cause could also explain the comparable low error measured for the control tests. Also, the collection in vials of the biogas produced was difficult with leaking occurring in some tests (data not shown).

For these reasons, we decided to run a second set of tests with higher reactor capacity (3.5L reactors volume). The larger volume is supposed to reduce the error of the test setting up, and it has been considered a better size even if necessarily the tests would be run without replicates.

Second screening BMP tests: The experiments were run in 3.5 L reactors, without replicates. The total duration of the tests was ten days. Pressure and temperature measures were automatically collected every hour with an automatic manometer (Keller Leo2 – CH).

A comparison of the max theoretical production for biogas and methane is shown in Table 4. Graphene, biochar and activated carbon resulted in an increased biogas production compared to their respective control. The percentage increase value is reported as ∆. The only material addition that resulted in a decrease in the biogas production is zeolite. As zeolite is the only material tested with no conductivity, this result is in agreement with the hypothesis of an electron transfer benefit.

The conductivity values order sees graphene, then PAC, biochar and last zeolite: the same order is observed for the increase in biogas production. Observing the values obtained for methane, zeolite confirms its position as last "increasing effect", while the remaining order is changed with activated carbon as first followed by graphene and biochar. The addition of zeolite resulted in a reduced (compared to the control) max methane production.

Table 4: Comparison of Ym values (theoretical maximum) of biogas and methane productions.

Materials	Δ% biogas	Δ% CH_4
Zeolite 10 g/L	-3	-10.7
Graphene 0.5 g/L	+32	+10.4
Biochar 10 g/L	+9.8	+5.3
PAC 20 g/L	+17.7	+22.5

5 Conclusions

A possible DIET effect on five different substrates has been evaluated in batch scale BMP tests. The small scale system showed a high error in replicates that was deemed too high to obtain meaningful observations. The choice for 120 mL tests were done after evaluating literature data: in fact as also reported recently in a review (Park et al. 2018), DIET mechanism evaluation have been carried out in tests with reactor working volumes ranging between 10ml to 2 Liters.

Due to the high error we decided to use higher capacity reactors (3.5 liters) that allow to reduce the error in the test set up. These data were more consistent and allowed to correlate the addition of conductive material with an increased biogas production.

The addition of conductive material resulted in an increased biogas and methane production. The increased biogas production reflects the conductivity values of the materials added meaning that the most conductive gave also the highest increase. Lin et al. (2017) reported an increase by 25% in biomethane yield and a 19.5% increase in peak biomethane production rate with graphene (1.0 g/L) and the results obtained in the present project are in agreement with the published data, even if with lower percentage increase (10.4%), but with a lower graphene concentration tested (0.5g/L). It is worth noting that the absolute values obtained during the tests were always lower than the ones reported by Lin et al. (2017). Other authors tested Lower than 1.0g/L concentrations (Tian et al. 2019): they reported that graphene (30 and 120mg/L) had significantly positive effects on methane production rate, which increased by 17.0% and 51.4%. Contrary with literature data no relationship has been observed between conductive material concentration and increased biogas production. This could be caused by the short testing time that excluded the involvement of the biofilm variable.

6 Outlook and next steps

The results obtained in this project evidenced the positive effect of the addition of conductive material. However due to the test settings no information on the long-term effects is available: in particular the biofilm growth and maturation has not been evaluated as well as the use of a different carbon source. Also no direct cause –effect relationship has been showed for the conductive material and the DIET mechanism: we proved *just* that an increase in biogas and methane has been observed. The tests were run with a digester sludge as inoculum (as carried out by literature), but working with mixed culture hinders the knowledge of the role of the active microorganisms: we cannot argue that taxa able to exploit the DIET process were present and active in our tests, we can just affirm that acetogens and methanogens were present and active. Working with pure cultures of taxa known to be able to exchange electrons by DIET, would answer the question.
Then there would be the issue of the maintenance of the selected-active -DIET culture in a waste rich environment in which the DIET capability would be a selective edge; and this could be investigated by comparing the microbial community profiles during the tests.

7 National and international cooperation

8 Communication

Bioenergieforschungstagung 09.05.2019 Ittigen

9 Publications

The following paper partially supported by the present grant has been published:

Principi P, König R, Cuomo M. Anaerobic digestion of lignocellulosic substrates: benefits of pre-treatments
Journal: Current Sustainable/Renewable Energy Reports DOI: 10.1007/s40518-019-00131-6

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