

Title	Boosting biomethane yield and production rate with graphene: the potential of direct interspecies electron transfer in anaerobic digestion
Authors	Lin, Richen;Cheng, Jun;Zhang, Jiabei;Zhou, Junhu;Cen, Kefa;Murphy, Jerry D.
Publication date	2017-05-05
Original Citation	Lin, R., Cheng, J., Zhang, J., Zhou, J., Cen, K. and Murphy, J. D. (2017) 'Boosting biomethane yield and production rate with graphene: The potential of direct interspecies electron transfer in anaerobic digestion', Bioresource Technology, 239, pp. 345-352. doi:10.1016/j.biortech.2017.05.017
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.biortech.2017.05.017
Rights	© 2017 Published by Elsevier Ltd. This manuscript version is made available under the CC-BY-NC-ND 4.0 license - http:// creativecommons.org/licenses/by-nc-nd/4.0/
Download date	2025-07-06 03:48:38
Item downloaded from	https://hdl.handle.net/10468/4060



University College Cork, Ireland Coláiste na hOllscoile Corcaigh

Accepted Manuscript

Boosting biomethane yield and production rate with graphene: the potential of direct interspecies electron transfer in anaerobic digestion

Richen Lin, Jun Cheng, Jiabei Zhang, Junhu Zhou, Kefa Cen, Jerry D. Murphy

PII:	S0960-8524(17)30656-9
DOI:	http://dx.doi.org/10.1016/j.biortech.2017.05.017
Reference:	BITE 18043
To appear in:	Bioresource Technology
Received Date:	13 March 2017
Revised Date:	30 April 2017
Accepted Date:	3 May 2017



Please cite this article as: Lin, R., Cheng, J., Zhang, J., Zhou, J., Cen, K., Murphy, J.D., Boosting biomethane yield and production rate with graphene: the potential of direct interspecies electron transfer in anaerobic digestion, *Bioresource Technology* (2017), doi: http://dx.doi.org/10.1016/j.biortech.2017.05.017

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1	Boosting biomethane yield and production rate with graphene: the
2	potential of direct interspecies electron transfer in anaerobic
3	digestion
4	
5	Richen Lin ^{a,b,c} , Jun Cheng ^{a,*} , Jiabei Zhang ^a , Junhu Zhou ^a , Kefa Cen ^a , Jerry D. Murphy ^{b,c}
6	^a State Key Laboratory of Clean Energy Utilization, Zhejiang University, Hangzhou 310027, China
7	^b MaREI Centre, Environmental Research Institute, University College Cork, Cork, Ireland
8	^c School of Engineering, University College Cork, Cork, Ireland
9	
10	Abstract
11	Interspecies electron transfer between bacteria and archaea plays a vital role in enhancing
12	energy efficiency of anaerobic digestion (AD). Conductive carbon materials (i.e. graphene
13	nanomaterial and activated charcoal) were assessed to enhance AD of ethanol (a key intermediate
14	product after acidogenesis of algae). The addition of graphene (1.0 g/L) resulted in the highest
15	biomethane yield (695.0 \pm 9.1 mL/g) and production rate (95.7 \pm 7.6 mL/g/d), corresponding to an
16	enhancement of 25.0% in biomethane yield and 19.5% in production rate. The ethanol degradation
17	constant was accordingly improved by 29.1% in the presence of graphene. Microbial analyses
18	revealed that electrogenic species of Geobacter and Pseudomonas along with archaea
19	Methanobacterium and Methanospirillum might participate in direct interspecies electron transfer
20	(DIET). Theoretical calculations provided evidence that graphene-based DIET can sustained a
21	much higher electron transfer flux than conventional hydrogen transfer.

^{*} Corresponding author: Prof. Dr. Jun Cheng, State Key Laboratory of Clean Energy Utilization, Zhejiang University, Hangzhou 310027, China. Tel.: +86 571 87952889; fax: +86 571 87951616. E-mail: juncheng@zju.edu.cn

Keywords: Graphene; activated charcoal; ethanol; direct interspecies electron transfer; anaerobic
 digestion.

R

3

4 **1. Introduction**

5	Anaerobic digestion (AD) of wet organic biomass for biogas production provides a
6	sustainable route to reduce fossil fuel use and greenhouse gas emissions, whilst producing
7	alternative dispatchable energy (Shen et al., 2015). The biogas industry developed rapidly in
8	Europe, in particular in Germany with 62% of the total biogas plants (Torrijos, 2016). Biogas
9	satisfied 4.7% of electricity and 1% of heat demand in Germany in 2014 (Torrijos, 2016). Due to
10	the high growth rate and carbohydrate content, algae including micro- and macro-algae are
11	considered to be a viable alternative energy feedstock that is devoid of the major drawbacks
12	associated with first and second-generation feedstock (Chen et al., 2015; Nigam & Singh, 2011).
13	However, AD of algae involving biological, chemical, and physical reactions can be limited by the
14	long retention time, low biodegradation efficiency, and low biogas production rate. The energy
15	conversion efficiency and process stability of AD can be easily disturbed by various biological and
16	environmental factors, such as process temperature, pH value, hydrodynamics, and organic
17	loading and detention time (Cheng & Call, 2016; Viggi et al., 2014).
18	The AD performance needs to be improved to make the process more economically viable.
19	Optimizations of the process control variables were reported effective to minimize energy
20	consumption and increase biogas production (Kusiak & Wei, 2012; Wei & Kusiak, 2012). Wei et
21	al developed a data-driven prediction model to optimize biogas production from sludge, in which
22	temperature, total solids, volatile solids and pH were employed as controllable variables. A 20.8%

1	increase was obtained when all controllable values were set to the optimal values (Wei & Kusiak,
2	2012). Many studies have focused on pretreatment development (such as thermal, mechanical,
3	chemical and biological methods) to overcome feedstock recalcitrance and enhance subsequent
4	AD performance (Ariunbaatar et al., 2014). The effects of various pretreatment methods are
5	highly different depending on the feedstock characteristics. Nevertheless, pretreatment methods
6	could be unsustainable in terms of environmental impacts, even if they enhance AD efficiency
7	(Ariunbaatar et al., 2014; Carballa et al., 2011).
8	From a biological perspective, AD is carried out by different groups of microorganisms
9	involved in hydrolysis, acidogenesis, acetogenesis and methanogenesis; interspecies electron
10	transfer between syntrophic bacteria and methanogenic archaea plays a vital role in enhancing AD
11	efficiency (Stams & Plugge, 2009). The predominant understanding for interspecies electron
12	transfer in AD was based on mediated interspecies electron transfer (MIET) via hydrogen or
13	formate (Rotaru et al., 2014b; Storck et al., 2016). MIET is normally endergonic under standard
14	conditions and is feasible only at very low metabolite concentration (especially hydrogen) due to
15	the thermodynamic constraints (Viggi et al., 2014). Recent findings revealed that direct
16	interspecies electron transfer (DIET) via electrically conductive pili, mineral, or shuttle molecules
17	is energetically more advantageous than MIET, because DIET does not require the multiple
18	enzymatic steps to produce hydrogen as an electron carrier (Lovley, 2011; Zhao et al., 2015).
19	Conductive materials (such as nano-magnetite, graphite, biochar, activated carbon and carbon
20	cloth) may avoid the energy consumption associated with the production of extracellular
21	conductive pili and associated c-type cytochromes for the provision of biological electrical
22	connections between cells (Kato et al., 2012; Liu et al., 2012; Zhao et al., 2015). Activated carbon

1	has been proven to promote DIET in AD of different types of substrates, such as ethanol,
2	propionate, butyrate and glucose (Lee et al., 2016; Zhao et al., 2016b). The biomethane production
3	rate in the presence of biochar increased by 16-25% in AD of propionate and butyrate (Zhao et al.,
4	2016a). Carbon-based nanomaterials exhibited the potential to stimulate DIET using glucose and
5	sucrose as substrates (Li et al., 2015; Tian et al., 2017). Tian et al. demonstrated that
6	methanogenesis of glucose was improved by addition of graphene during long-term anaerobic
7	digestion under low temperature (10-20 °C). Despite the establishment of DIET in pure and mixed
8	cultures, the application of nano-scale carbonaceous materials in traditional AD requires further
9	investigations to improve the AD performance.
10	As a highly-conductive nanomaterial, graphene has received heightened attention for
11	biotechnological applications, such as electrode materials in microbial fuel cells (ElMekawy et al.,
12	2016; Perreault et al., 2015). Graphene has known antimicrobial properties in some cases
13	(Catherine et al., 2012; Nguyen et al., 2017), however, little is known about the impact of
14	graphene on anaerobic microbial communities in AD. The unique physicochemical properties of
15	graphene, notably its exceptionally high electric conductivity, large surface area and good
16	mechanical strength, may provide a solution to improve the stability and efficiency of AD.
17	Therefore, it is hypothesized that graphene can significantly facilitate DIET and enhance AD
18	efficiency. However, to the best of our knowledge, the research of DIET in AD of ethanol in the
19	presence of graphene is rather sparse. Theoretical comparison of electron transfer flux between
20	graphene-based DIET and MIET have not been calculated previously. The interactions between
21	nanomaterial and microbes in AD have not been revealed. In this study, ethanol was used as
22	feedstock to investigate DIET in AD, as ethanol is a key intermediate product after acidogenesis of

1	algae feedstock (accounting for 15.6%-34.2% of total energy production (Xia et al., 2015; Xia et
2	al., 2016)). The innovation and objectives of this study are as follows: (1) Compare the kinetics of
3	biomethane production with different additions of graphene and activated charcoal in AD of
4	ethanol; (2) Identify the bacterial and archaeal communities responsible for graphene-based DIET
5	in AD; (3) Calculate the maximum electron transfer flux of MIET and graphene-based DIET for
6	the first time.
7	5
8	2. Materials and methods
9	2.1. Inoculum and materials
10	The inoculum was sourced from a laboratory digester mainly treating cellulose. Graphene and
11	activated charcoal were both purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd,
12	China. The size of activated charcoal was approximately 10-32 mesh (equivalent to 0.5-1.7 mm).
13	The micro size of graphene was around $5\sim10$ µm, and the thickness of graphene nanoplatelets was
14	between 4-20 nm. More details on physic-chemical properties of graphene are available in
15	http://www.aladdin-e.com/up_files/docs/G139804.pdf.
16	
17	2.2. Experimental design

18 Batch experiments of AD were conducted in glass fermenters (300 mL working volume). Each

- 19 bottle contained 2.5 mL of ethanol as feedstock and 250 mL of activated sludge as inoculum. The
- 20 initial pH was adjusted to 7.5 ± 0.1 through use of 6 M HCl and 6 M NaOH solution.
- 21 Subsequently different amounts of graphene and activated charcoal were separately added into the
- 22 glass bottles. The concentrations of graphene were set as 0, 0.5, 1.0 and 2.0 g/L. Considering the

1	electric conductivity of activated charcoal is much lower than that of graphene, the concentrations
2	of activated charcoal were set as 0, 5, 10, 20 and 30 g/L. Deionized water was added to adjust the
3	total solution to 300 mL. Afterwards, all the bottles were sealed with rubber stoppers, purged with
4	nitrogen gas for 10 min, and maintained at 35 \pm 1.0 °C during AD. The produced biogas and liquid
5	solution during methanogenesis were sampled and analyzed at an interval of 2 d. The pH value of
6	solutions was readjusted to 7.5 every 2 d in order to prevent a severe pH drop during AD. All the
7	experiments were conducted in duplicate.
8	
9	2.3. Microbial community analysis
10	A volume of 5ml of anaerobic sludge was collected from the bottom of the reactors after the AD
11	experiments. The sludge samples were rinsed with phosphate-buffered saline and then centrifuged
12	for 10 min at 4 °C. The pretreated samples were stored at -20 °C until further use. The microbial
13	community was characterized using high-throughput 16S rRNA pyrosequencing. DNA extraction
14	was performed following the manufacturer's protocol
15	(http://omegabiotek.com/store/wp-content/uploads/2013/04/D5625-Soil-DNA-Kit-101216-online-
16	1.pdf). The DNA samples were amplified in two independent PCR reactions with primers
17	spanning the V3-V4 hypervariable region of the 16S rRNA gene. PCR products were checked in 2%
18	agarose gel to determine the success of amplification. Samples were pooled together in equal
19	proportions based on their molecular weight and DNA concentrations. Then the samples were
20	purified using calibrated Ampure XP beads. The pooled and purified PCR product was used to
21	prepare the DNA library by following Illumina TruSeq DNA library preparation protocol.
22	Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq

1	following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis
2	pipeline (MR DNA, Shallowater, TX, USA). Operational taxonomic units (OTUs) were defined
3	by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using
4	BLASTN against a curated database derived from GreenGenes, RDPII and NCBI
5	(www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) (DeSantis et al., 2006).
6	
7	2.4. Microscope observation
8	The microbial morphology of sludge in response to graphene was observed on a field emission
9	scanning electronic microscope (SEM, Hitachi SU 8010, Japan) (Cheng et al., 2013). The samples
10	from the reactors were fixed with 2.5% (v/v) glutaral dehyde at 4 °C, washed followed by stepwise
11	dehydration in a gradient series of ethanol solutions and then CO ₂ critical point dried. Samples
12	were finally coated with gold and observed by SEM.
13	
14	2.5. Analytical methods
15	The concentrations of biomethane and carbon dioxide were analyzed on a gas chromatography
16	system (GC; Agilent 7820A, USA) equipped with a thermal conductivity detector and a 5A
17	column. The concentrations of ethanol and acetate were analyzed on another GC system equipped
18	with a flame ionization detector and a DB-FFAP column (Lin et al., 2016).
19	Biomethane yield was simulated by the modified Gompertz equation (Eq. 1), and kinetic
20	parameters (H_m , maximum methane yield potential, mL/g ethanol; R_m , peak methane production
21	rate, mL/g ethanol/h; λ , lag-phase time of methane production, h; and T_m , peak time of methane
22	fermentation, h) were calculated using Origin 8.5 software.

1	$H = H_m \exp\left\{-\exp\left[\frac{R_m e}{H_m}(\lambda - t) + 1\right]\right\}$	(1)
2	The degradation of ethanol was assumed to fit a first-order model (Eq. 2), where C_e is ethanol	
3	concentration (mM), C_{e0} is the initial ethanol concentration (mM), and k_e is the ethanol degradation	rate
4	constant (d ⁻¹).	2
5	$C_e = C_{e0} \exp\left(-k_e t\right)$	(2)
6	The overall electron recovery after AD of ethanol was calculated according to Eq. 3.	
7	Electron revovery $\% = \frac{\text{Actual biomethane yield}}{\text{Stoichiometric conversion of ethanol to biomethane}} \times 100\%$ (3)	
8		
9	3. Results and discussion	
10	3.1 Effects of conductive materials on biomethane production kinetics in	
11	anaerobic digestion	
12	To evaluate the effects of the conductive materials (graphene and activated charcoal) on the	ne
13	performance of AD, ethanol (a low-molecular substrate) was used as a model carbon source. T	he
14	effects of graphene and activated charcoal on biomethane yield and production rate are shown	in
15	Fig. 1 a and b. The biomethane yield from ethanol without conductive material addition was	
16	556.1 \pm 53.3 mL/g after 12 d. The peak biomethane production rate was obtained as 80.1 \pm 0.2	
17	mL/g/d at 4 d. The addition of 20.0 g/L of activated charcoal gave a maximum biomethane yie	ld
18	of 627.2 ± 30.7 mL/g. The peak biomethane production rate was obtained as 91.1 ± 18.6 mL/g/d.	
19	The biomethane yield and peak biomethane production rate were greatly enhanced by 12.8% a	nd
20	13.7%, respectively. The biomethane yields achieved with 5.0, 10.0 and 30.0 g/L of activated	
21	charcoal were lower than that with 20.0 g/L of activated charcoal (data were not shown in Fig.	1 in
22	order to make it more explicit). Therefore, the optimal concentration of 20.0 g/L activated	

1	charcoal was used for further comparison with different concentrations of graphene. It was proven
2	that carbonaceous materials such as graphite, biochar, and carbon cloth are capable of promoting
3	methane fermentation and chemical oxygen demand removal (Zhao et al., 2015). Li et al. reported
4	that the electrical conductance of the sludge was enhanced in the presence of carbon nanotube,
5	which might promote DIET among fermentative bacteria and methanogens in the AD process (Li
6	et al., 2015). Thus, it is hypothesized that materials with higher conductivity may play a more
7	significant role in promoting DIET. Fig. 1 shows that the biomethane yield positively increased
8	from 556.1±53.3 (0 g/L graphene) to 670.9±16.0 (0.5 g/L graphene), 695.0±9.1 (1.0 g/L graphene)
9	and 662.9 \pm 14.7 mL/g (2.0 g/L graphene). The optimal concentration of graphene (1.0 g/L)
10	resulted in a 25.0% increase in biomethane yield and a 19.5% increase in peak biomethane
11	production rate. However, on further increasing the graphene concentration to 2.0 g/L, the
12	biomethane yield slightly decreased to 662.9±14.7 mL/g. This result was probably ascribed to the
13	microbial inhibition effect by the high concentration of graphene, indicating that cytotoxicity
14	could become a limiting factor when applying nanomaterials in AD. Cytotoxicity of carbon
15	nanomaterials (such as graphene and carbon nanotube) to microbes has been demonstrated using
16	different microbial strains such as Escherichia coli and Bacillus subtilis (Liu et al., 2011; Pasquini
17	et al., 2012; Zhu et al., 2014). The toxicological molecular mechanisms of nanomaterials remained
18	limited, but a possible explanation was related to the synergistic impacts of cell membrane
19	perturbation and oxidative stress (Qu et al., 2015).
20	It was noted that the optimal graphene addition resulted in a more significant enhancement of
21	biomethane production as compared to the optimal activated charcoal addition (even with a
22	concentration of 20.0 g/L). This result can be ascribed to the following reasons: (1) The electrical

1	conductivity of graphene is much higher than that of activated charcoal, resulting in higher
2	electron transfer efficiency in DIET; (2) The micro size of graphene is much smaller, resulting in a
3	higher specific surface area and a better interaction with microbes.
4	The kinetic parameters of biomethane production fitted by the modified Gompertz equation
5	are shown in Table 1. The kinetics of biomethane production were evaluated in terms of the
6	biomethane yield potential (H_m), peak biomethane production rate (R_m), lag phase time (λ) and
7	peak time (T_m). The maximum biomethane yield potential (H_m , 718.4 mL/g) was achieved in the
8	presence of 1.0 g/L graphene, corresponding to a value of 23.4% higher than the control.
9	Accordingly, the peak biomethane production rate (R_m , 116.2 mL/g/h) was obtained with the
10	addition of 1.0 g/L graphene, corresponding to a value of 33.7% higher than the control. The lag
11	phase time (λ) and peak time (T_m) both reduced to a great extent in the presence of graphene. On
12	comparison to graphene, the biomethane production performance in terms of biomethane yield
13	potential and peak biomethane production rate was less enhanced with addition of activated
14	charcoal, even at a high concentration of 20.0 g/L.
15	
16	3.2 Effects of conductive materials on ethanol degradation in anaerobic digestion
17	The complete conversion of ethanol to biomethane in AD requires the combined acetogenic
18	bacteria and methanogenic archaea mediating the three step reactions (Table 2). Acetogenic
19	bacteria are responsible for converting ethanol into acetate and releasing electrons. Methanogenic
20	archaea are responsible for converting produced acetate, carbon dioxide and electron into
21	biomethane. The effects of graphene and activated charcoal on degradation of ethanol are shown
22	in Fig. 2a. It was observed that ethanol was continuously consumed by acetogenic bacteria during

1	12 d of AD. Ethanol degradation was more rapid with the addition of conductive materials. In the
2	presence of 1.0 g/L graphene and 20.0 g/L activated charcoal, 50.3% and 43.5% of ethanol were
3	consumed in the first 2d of AD. Comparatively, only 32.0% of ethanol was consumed in the
4	absence of conductive materials. Apparently, graphene played a significant role in rapid
5	degradation of ethanol, which is possibly due to the high electrical conductivity and specific
6	surface area. The ethanol degradation rate constants derived from the first-order equation are
7	shown in Fig. 2b. In the absence of conductive materials, the calculated ethanol degradation rate
8	constant was only 0.31 \pm 0.03 /d. This value increased to 0.37 \pm 0.04 /d and 0.40 \pm 0.03 /d with the
9	addition of activated charcoal and graphene, respectively. The higher ethanol degradation rate
10	constant indicated that the substrate degradation in AD can be significantly enhanced through the
11	presence of conductive materials.
12	With continuous degradation of ethanol, acetate reached a peak concentration, and was
13	subsequently depleted by aceticlastic methanogen (Fig. 2c). The highest acetate concentration of
14	58.1 mM was obtained at 6 d in the presence of 1.0 g/L graphene, as compared to acetate
15	concentration of 43.4 mM in the absence of conductive materials. Ethanol and acetate were
16	completely consumed at the end of AD (12 d). It was clearly observed that acetate generation by
17	acetogenic bacteria and subsequent consumption by aceticlastic methanogens were much faster in
18	the presence of graphene, resulting in promoted methanogenesis and enhanced biomethane yield.
19	These result indicated that conductive materials are capable of promoting syntrophic reactions
20	between acetogenic bacteria and methanogenic archaea, which facilitates substrate degradation
21	and utilization for enhanced methanogenesis.

22 To evaluate the overall efficiency of ethanol conversion in AD, the electron recovery was

1	calculated in terms of biomethane yield and stoichiometric conversion of ethanol to biomethane
2	(Fig. 2d). Consistent with the biomethane yield, the highest electron recovery of $95.1 \pm 1.2\%$ was
3	achieved in the presence of graphene, as compared to 76.1±7.3% in control group. The enhanced
4	electron recovery was attributed to enhanced DIET efficiency with graphene. It was noteworthy
5	that 100% conversion of ethanol to biomethane was almost impossible. This was mainly because
6	the biodegradation of ethanol in AD not only generates acetate (Table 2) but also minor amounts
7	of propionate, butyrate, or caproate along with the consumption of hydrogen. In addition, some
8	energy derived from ethanol degradation is required to support microbial growth.
9	
10	3.3 Microbial community analyses after anaerobic digestion
11	3.3.1. Bacterial community composition
12	The enhanced biomethane production was highly dependent on the syntrophic activities of
13	electron-producing acetogens and electron-consuming methanogens. The changes in the microbial
14	community might provide a clue to the reason for enhanced biomethane production. Bacterial and
15	archaeal community structures at genus level after AD of ethanol are shown in Table 3 and 4. The
16	taxonomic compositions of the initial inoculum were distinctly different from those fed with
17	ethanol after AD. This result is ascribed to the assimilation effect as ethanol is the only carbon
18	source, which selectively enriched strains favoring ethanol utilization.
19	In the original inoculum, <i>Clostridium</i> (10.1%), <i>Levilinea</i> (7.6%), and <i>Aminobacterium</i> (4.0%)
20	were the three major bacterial genera. Clostridium are metabolically versatile(Lee et al., 2007) and
21	are the predominant strains involved in dark hydrogen fermentation converting carbohydrates to
22	hydrogen along with the production of volatile fatty acids (VFAs, such as acetate and butyrate).

1	Levilinea are recognized as an anaerobic fermentative bacterium, which ferment sugars and amino
2	acids into hydrogen, acetic and lactic acids.(Yamada et al., 2006) Aminobacterium are capable of
3	degrading amino acids to VFAs (Baena et al., 1998).
4	After the AD of ethanol without graphene, the dominant bacterial groups shifted to Levilinea
5	(11.6%), <i>Clostridium</i> (8.6%), and <i>Geobacter</i> (8.4%). The abundance of amino acid-degrading
6	Aminobacterium decreased to 2.1%, which can be ascribed to the absence of amino acids during
7	AD. Geobacter were enriched from 0.3% (in initial inoculum) to 8.4% with ethanol as substrate. A
8	variety of Geobacter species (such as G. metallireducens, G. pickeringii, and G. lovleyi) has the
9	ability to utilize ethanol as an electron donor to support their growth metabolism (Lovley et al.,
10	2011).
11	With the addition of graphene in AD, the dominant bacterial groups shifted to Geobacter
12	(9.9%), Pseudomonas (6.9%) and Levilinea (6.2%). The abundance of Geobacter increased to 9.9%
13	in the presence of graphene, as compared to 8.4% without graphene. The high electrical
14	conductivity of graphene may contribute to the shift during AD. Graphene enhances the electron
15	transfer during ethanol degradation by Geobacter, which in turn facilitates the growth of
16	Geobacter. Geobacter are well-known iron-respiring bacteria and are distributed widely in
17	anaerobic environments; they are among the most effective microorganisms for harvesting
18	electrical current from organic compounds (Lovley et al., 2011). It has been reported that
19	Geobacter play a significant role in performing DIET either directly through extracellular pili or
20	using additional conductive materials (Cheng & Call, 2016). DIET was first documented in
21	co-culture of Geobacter species, where Geobacter metallireducens is capable of transferring
22	electrons derived from ethanol to the partner Geobacter sulfurreducens via electrically conductive

1	pili (Summers et al., 2010). DIET was also recorded in anaerobic digesters where Geobacter
2	transfer electrons to Methanosaeta (Rotaru et al., 2014b). DIET may yield more energy than
3	conventional MIET because there is less energy loss associated with the formation of
4	intermediates and the subsequent reactions needed to oxidize them (Lovley, 2011; Zhao et al.,
5	2015). This could be one plausible explanation for the acceleration of methanogenesis in the
6	presence of graphene. Therefore, it is concluded that the predominance of Geobacter population
7	(9.9%) found in the presence of graphene is a potent support for DIET in AD of ethanol. It was
8	also found the abundance of <i>Pseudomonas</i> was greatly increased to 6.9% in the presence of
9	graphene, as compared to only 1.9% without graphene. Pseudomonas species are recognized as
10	electrogenic bacteria responsible for converting VFAs to electric current in microbial fuel cells
11	(Freguia et al., 2010). Pseudomonas are also capable of converting ethanol to acetate along with
12	the production of electrons. However, it was reported that <i>Pseudomonas</i> are unable to effectively
13	transfer electrons derived from central metabolism to the outside of the cell (Lovley, 2006). It was
14	demonstrated that <i>Pseudomonas aeruginosa</i> yielded poorly conductive pili (Reguera et al., 2005),
15	which cannot be used as conduit for extracellular electron transfer. The addition of graphene in
16	AD could act as an alternative to conductive pili and an aid in electron transfer from Pseudomonas
17	to the methanogenic partners during AD, contributing to the enhanced growth of <i>Pseudomonas</i> . As
18	a result, since the electrogenic bacteria of Geobacter and Pseudomonas species were found greatly
19	enriched with the addition of graphene, they are proposed to be responsible for DIET in AD of
20	ethanol.

21

22 3.3.2. Archaeal community composition

1	The archaeal community structures at genus level with/without graphene addition after AD of
2	ethanol are shown in Table 4. The majority of archaeal communities were mainly comprised of 4
3	archaeal genera. In the original inoculum, Methanosaeta were the most dominant species
4	accounting for 86.1% of the total abundance, followed by <i>Methanolinea</i> (6.4%),
5	Methanobacterium (2.7%) and Methanospirillum (1.1%). Methanosaeta are often abundant in
6	anaerobic digesters and are mainly acetate-consuming methanogens which cleave acetate into CH4
7	and CO ₂ (Table 2). <i>Methanosaeta</i> are also capable of receiving electrons via DIET for CO ₂
8	reduction into CH ₄ (Rotaru et al., 2014b). Methanolinea, Methanobacterium and
9	Methanospirillum are conventionally recognized as hydrogen-consuming methanogens, which
10	convert CO_2 and H_2 into CH_4 (Table 2).
11	After AD of ethanol, the dominant archaeal groups were greatly changed due to the
12	acclimatization effect by ethanol. With the addition of graphene in AD, the dominant archaeal
13	groups shifted to Methanosaeta (39.8%), Methanobacterium (34.9%) and Methanolinea (9.8%). It
14	was noted that the abundance of Methanosaeta decreased to 39.8% in the presence of graphene as
15	compared to 50.1% without graphene, suggesting that the pathway of CH_4 production by acetate
16	cleavage was weakened. The abundance of Methanolinea decreased from 20.2% to 9.8% with the
17	addition of graphene. Comparatively, Methanobacterium became more predominant, increasing
18	from 24.0% to 34.9%, while Methanospirillum were enriched from 2.2% to 7.8%. The shift of
19	archaeal structures indicated the metabolic pathway was changed in AD in the presence of
20	graphene. To date, Methanosarcina and Methanosaeta species are the only methanogens known to
21	participate in DIET by directly receiving electrons to reduce CO ₂ into CH ₄ (Rotaru et al., 2014a;
22	Rotaru et al., 2014b). However, in this study the enrichment of Methanobacterium and

1	Methanospirillum	with the additio	n of graphene	proposes a	possibility	that they	z may pla	v an
-	mennospininni	with the additio	n or gruphene	proposes u	possionity	that the	ina pia	.y un

2 important role in performing DIET.

3	The communities of bacteria and archaea observed in the presence of graphene provided a
4	clue for the reason of enhanced biomethane production and also provided a mechanistic
5	explanation. Taken together, electrogenic bacteria of Geobacter and Pseudomonas species along
6	with archaea Methanobacterium and Methanospirillum may participate in DIET in AD of ethanol,
7	contributing to the enhanced AD performance.
8	
9	3.4 Effects of conductive materials on microbial morphologies after anaerobic
10	digestion
11	Microbial morphologies after AD with/without graphene addition are shown in Fig. S1 in the
12	Supplementary material. Rod-shaped cells with different lengths of 1-4 μ m are predominant in
13	both samples with/without graphene addition. It appears that cells are attached together (Fig. S1 c
14	and d), forming microbial aggregates after digestion in the presence of graphene. The direct
15	contact of cells in aggregates may allow direct electron transfer during AD. It is also observed that
16	there are extracellular "microbial nanowires" (~50 nm) formed on cell surfaces, exhibiting typical
17	characteristic of electrogenic bacteria (such as Geobacter). However, it is unknown if these
18	microbial structures are electrically conductive. It was reported that the aggregates in the
19	anaerobic reactors treating brewery wastes exhibited a high and metal-like conductance (Morita et
20	al., 2011; Shrestha et al., 2014). The conductive property of aggregates was ascribed to that
21	Geobacter species, which produce electrically conductive pili with a high and metal-like
22	conductance (Morita et al., 2011).

1	
2	3.5 Theoretical analysis of graphene-based direct interspecies electron transfer
3	and interspecies hydrogen transfer
4	Interspecies electron transfer in AD can rely on either DIET (between electron-producing
5	acetogens and electron-producing methanogens) or MIET with hydrogen as electron carrier. The
6	simplified electron transfer mechanisms for DIET and MIET are illustrated in Fig. 3. To
7	quantitatively compare the electron transfer efficiencies of DIET and MIET, the theoretical
8	maximum electron carrier fluxes were calculated based on Nernst equation and Fick's diffusion
9	law (Mao et al., 2015; Viggi et al., 2014). The detailed parameters for calculations were provided
10	in the Supplementary material.
11	The maximum electron flux for the graphene-based DIET was calculated as following.
12	Assuming that the electrons are released from ethanol degradation through electron-donating
13	reaction (Table 2, $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 5H^+ + 4e^-, \Delta G^{0} = -149.64 \text{ kJ/mol}$), then the
14	electrons are directly transferred to methanogens via graphene. Methanogens reduce CO ₂ to CH ₄
15	through electron-consuming reaction (Table 2, $4H^+ + 4e^- + 1/2CO_2 \rightarrow 1/2CH_4 + H_2O$, $\Delta G^{0} = 93.98$
16	kJ/mol). The maximum driving force for electron transfer is given by the redox potential (ΔE) of
17	the overall reaction (CH ₃ CH ₂ OH + $1/2$ CO ₂ $\rightarrow 1/2$ CH ₄ + CH ₃ COO ⁻ + H ⁺ , Δ G ⁰ /= -55.67 kJ/mol),
18	which was calculated as 0.136 V. The resulting maximum electron flux via graphene was
19	determined as approximately 7×10^{-4} A (see calculations in Fig. S2 in the Supplementary
20	material).
21	To estimate the maximum H_2 flux in MIET, the concentrations of reactants and products were

set identical to those in DIET. Fick's law is used to compute the rate of H_2 diffusion from the

1	acetogen to the methanogen (Mao et al., 2015). The maximum driving force for H_2 diffusion
2	depends on the highest H_2 concentration generated by acetogens and the lowest H_2 concentration
3	reached by methanogens. The highest H ₂ concentration was calculated in terms of the
4	electron-donating reaction (Table 2, $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$, corresponding
5	to $\Delta G' = 0$), and the lowest H ₂ concentration was calculated in terms of the electron-consuming
6	reaction (Table 2, $2H_2 + 1/2CO_2 \rightarrow 1/2CH_4 + H_2O$, corresponding to $\Delta G' = 0$). The obtained
7	highest and lowest H_2 concentrations were approximately 70.8 μ M and 1.5 nM, respectively.
8	Therefore, a maximum H_2 flux was achieved as approximately 3.6×10^{-6} nmol/s, theoretically
9	corresponding to an equivalent electric current of 7×10^{-10} A (see calculations in Fig. S3 in the
10	Supplementary material).
11	Clearly there is a huge difference in maximum electron transfer rate between graphene-based
12	DIET and MIET via hydrogen. The maximum electron flux calculated in DIET is theoretically
13	around 10^6 times higher than that obtained in MIET. The calculations also give a clue that
14	activated charcoal may not be effective to conduct DIET due to its poor electrical conductivity (~5
15	orders of magnitude lower than graphene (Adinaveen et al., 2016). It should be pointed out that
16	several assumptions were made to calculate the electron fluxes, such as the microbial cell shape,
17	average distance between cells, graphene shape, no energy loss as heat during electron transfer,
18	and no energy consumption for microbial growth. The driving force for electron transfer is
19	assumed to be totally determined by Gibbs free energy ($\Delta G' = 0$), which does not consider the
20	energy conserved for microorganism growth and energy loss as heat during electron transfer.
21	However, a free energy of about -20 to -15 kJ/mol is normally required to support syntrophic
22	microbial growth (Schink, 1997). These assumptions will undoubtedly result in the inaccuracy on

1	the final calculated value for MIET and DIET, which necessitate a more accurate model
2	development. However, in spite of these considerations, the computed difference between MIET
3	and DIET is so large that the kinetic advantage of the DIET via graphene is apparent. The result
4	provides evidence that graphene-based DIET can intrinsically sustain electric current flux up to 6
5	orders of magnitude than that of hydrogen-based MIET, allowing for more efficient electron
6	transfer in syntrophic mechanism in AD of ethanol.
7	9
8	Conclusions
9	Highly-conductive graphene was able to stimulate DIET to boost biomethane yield and
10	production rate from ethanol. The addition of 1.0 g/L graphene resulted in an enhancement of 25.0%
11	in biomethane yield and 19.5% in production rate. The degradation rate of ethanol was
12	simultaneously enhanced. Electrogenic bacteria of Geobacter and Pseudomonas species along
	with an hard Made and Made and Million wight and interest in DIFT and an it's

- 13 with archaea *Methanobacterium* and *Methanospirillum* might participate in DIET responsible for
- 14 enhanced AD performance. Graphene-based DIET intrinsically sustained a much higher electron
- 15 transfer flux than conventional hydrogen transfer. Reutilization of conductive materials should be

16 considered to make DIET-based AD economically viable.

17

18 Acknowledgements

- 19 This collaborative Chinese Irish study was supported by the National key research and
- 20 development program-China (2016YFE0117900), National Natural Science Foundation-China
- 21 (51676171), Zhejiang Provincial Key Research and Development Program-China (2017C04001),
- 22 and also funded by Science Foundation Ireland (SFI) through the Centre for Marine and

1	Renew	able Energy (MaREI) under Grant No. 12/RC/2302. The work was also co-funded by Gas
2	Netwo	rks Ireland (GNI) through the Gas Innovation Group, and by ERVIA.
3		
4	Refe	rences
5	[1]	Adinaveen, T., Vijaya, J.J., Kennedy, L.J. 2016. Comparative Study of Electrical
6		Conductivity on Activated Carbons Prepared from Various Cellulose Materials. Arabian
7		Journal for Science and Engineering, 41 , 55-65.
8	[2]	Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F., Lens, P.N.L. 2014. Pretreatment
9		methods to enhance anaerobic digestion of organic solid waste. Applied Energy, 123,
10		143-156.
11	[3]	Baena, S., Fardeau, M.L., Labat, M., Ollivier, B., Thomas, P., Garcia, J.L., Patel, B.K.C.
12		1998. Aminobacterium colombiensegen. nov. sp. nov., an Amino Acid-degrading
13		Anaerobe Isolated from Anaerobic Sludge. Anaerobe, 4, 241-250.
14	[4]	Carballa, M., Duran, C., Hospido, A. 2011. Should We Pretreat Solid Waste Prior to
15		Anaerobic Digestion? An Assessment of Its Environmental Cost. Environmental Science
16		& Technology, 45 , 10306-10314.
17	[5]	Catherine, M.S., Joey, M., Farid, A., Alex, L., Rigoberto, C.A., Debora, F.R. 2012.
18		Graphene nanocomposite for biomedical applications: fabrication, antimicrobial and
19		cytotoxic investigations. Nanotechnology, 23, 395101.
20	[6]	Chen, G., Zhao, L., Qi, Y. 2015. Enhancing the productivity of microalgae cultivated in
21		wastewater toward biofuel production: A critical review. Applied Energy, 137, 282-291.
22	[7]	Cheng, J., Sun, J., Huang, Y., Feng, J., Zhou, J., Cen, K. 2013. Dynamic microstructures

1		and fractal characterization of cell wall disruption for microwave irradiation-assisted lipid
2		extraction from wet microalgae. Bioresource Technology, 150, 67-72.
3	[8]	Cheng, Q., Call, D.F. 2016. Hardwiring microbes via direct interspecies electron transfer:
4		mechanisms and applications. Environmental Science: Processes & Impacts, 18, 968-980.
5	[9]	DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T.,
6		Dalevi, D., Hu, P., Andersen, G.L. 2006. Greengenes, a Chimera-Checked 16S rRNA
7		Gene Database and Workbench Compatible with ARB. Applied and Environmental
8		Microbiology, 72 , 5069-5072.
9	[10]	ElMekawy, A., Hegab, H.M., Losic, D., Saint, C.P., Pant, D. 2016. Applications of
10		graphene in microbial fuel cells: The gap between promise and reality. Renewable and
11		Sustainable Energy Reviews.
12	[11]	Freguia, S., Teh, E.H., Boon, N., Leung, K.M., Keller, J., Rabaey, K. 2010. Microbial fuel
13		cells operating on mixed fatty acids. Bioresource Technology, 101, 1233-1238.
14	[12]	Kato, S., Hashimoto, K., Watanabe, K. 2012. Methanogenesis facilitated by electric
15		syntrophy via (semi)conductive iron-oxide minerals. Environmental Microbiology, 14,
16		1646-1654.
17	[13]	Kusiak, A., Wei, X. 2012. Optimization of the activated sludge process. Journal of Energy
18		Engineering, 139 , 12-17.
19	[14]	Lee, J.Y., Lee, S.H., Park, H.D. 2016. Enrichment of specific electro-active
20		microorganisms and enhancement of methane production by adding granular activated
21		carbon in anaerobic reactors. Bioresour Technol, 205, 205-12.
22	[15]	Lee, YJ., Romanek, C.S., Wiegel, J. 2007. Clostridium aciditolerans sp. nov., an

1		acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment.
2		International Journal of Systematic and Evolutionary Microbiology, 57, 311-315.
3	[16]	Li, L.L., Tong, Z.H., Fang, C.Y., Chu, J., Yu, H.Q. 2015. Response of anaerobic granular
4		sludge to single-wall carbon nanotube exposure. <i>Water Research</i> , 70 , 1-8.
5	[17]	Lin, R., Cheng, J., Ding, L., Song, W., Liu, M., Zhou, J., Cen, K. 2016. Enhanced dark
6		hydrogen fermentation by addition of ferric oxide nanoparticles using Enterobacter
7		aerogenes. Bioresource Technology, 207, 213-219.
8	[18]	Liu, F.H., Rotaru, A.E., Shrestha, P.M., Malvankar, N.S., Nevin, K.P., Lovley, D.R. 2012.
9		Promoting direct interspecies electron transfer with activated carbon. Energy &
10		Environmental Science, 5, 8982-8989.
11	[19]	Liu, S., Zeng, T.H., Hofmann, M., Burcombe, E., Wei, J., Jiang, R., Kong, J., Chen, Y.
12		2011. Antibacterial Activity of Graphite, Graphite Oxide, Graphene Oxide, and Reduced
13		Graphene Oxide: Membrane and Oxidative Stress. ACS Nano, 5, 6971-6980.
14	[20]	Lovley, D.R. 2006. Bug juice: harvesting electricity with microorganisms. Nat Rev
15		<i>Microbiol</i> , 4 , 497-508.
16	[21]	Lovley, D.R. 2011. Live wires: direct extracellular electron exchange for bioenergy and
17		the bioremediation of energy-related contamination. Energy & Environmental Science, 4,
18		4896-4906.
19	[22]	Lovley, D.R., Ueki, T., Zhang, T., Malvankar, N.S., Shrestha, P.M., Flanagan, K.A.,
20		Aklujkar, M., Butler, J.E., Giloteaux, L., Rotaru, A.E., Holmes, D.E., Franks, A.E.,
21		Orellana, R., Risso, C., Nevin, K.P. 2011. Geobacter: the microbe electric's physiology,
22		ecology, and practical applications. Adv Microb Physiol, 59, 1-100.

1	[23]	Mao, X., Stenuit, B., Polasko, A., Alvarez-Cohen, L. 2015. Efficient Metabolic Exchange
2		and Electron Transfer within a Syntrophic Trichloroethene-Degrading Coculture of
3		Dehalococcoides mccartyi 195 and Syntrophomonas wolfei. Applied and Environmental
4		Microbiology, 81, 2015-2024.
5	[24]	Morita, M., Malvankar, N.S., Franks, A.E., Summers, Z.M., Giloteaux, L., Rotaru, A.E.,
6		Rotaru, C., Lovley, D.R. 2011. Potential for Direct Interspecies Electron Transfer in
7		Methanogenic Wastewater Digester Aggregates. mBio, 2, e00159-11.
8	[25]	Nguyen, H.N., Castro-Wallace, S.L., Rodrigues, D.F. 2017. Acute toxicity of graphene
9		nanoplatelets on biological wastewater treatment process. Environmental Science: Nano,
10		4 , 160-169.
11	[26]	Nigam, P.S., Singh, A. 2011. Production of liquid biofuels from renewable resources.
12		Progress in Energy and Combustion Science, 37, 52-68.
13	[27]	Pasquini, L.M., Hashmi, S.M., Sommer, T.J., Elimelech, M., Zimmerman, J.B. 2012.
14		Impact of surface functionalization on bacterial cytotoxicity of single-walled carbon
15		nanotubes. Environ. Sci. Technol., 46, 6297.
16	[28]	Perreault, F., Fonseca de Faria, A., Elimelech, M. 2015. Environmental applications of
17		graphene-based nanomaterials. Chem Soc Rev, 44, 5861-5896.
18	[29]	Qu, Y., Ma, Q., Deng, J., Shen, W., Zhang, X., He, Z., Van Nostrand, J.D., Zhou, J., Zhou,
19		J. 2015. Responses of microbial communities to single-walled carbon nanotubes in
20		phenol wastewater treatment systems. Environ Sci Technol, 49, 4627-35.
21	[30]	Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., Lovley, D.R. 2005.
22		Extracellular electron transfer via microbial nanowires. Nature, 435, 1098-101.

1	[31]	Rotaru, A.E., Shrestha, P.M., Liu, F., Markovaite, B., Chen, S., Nevin, K.P., Lovley, D.R.
2		2014a. Direct Interspecies Electron Transfer between Geobacter metallireducens and
3		Methanosarcina barkeri. Applied and Environmental Microbiology, 80, 4599-4605.
4	[32]	Rotaru, A.E., Shrestha, P.M., Liu, F.H., Shrestha, M., Shrestha, D., Embree, M., Zengler,
5		K., Wardman, C., Nevin, K.P., Lovley, D.R. 2014b. A new model for electron flow during
6		anaerobic digestion: direct interspecies electron transfer to Methanosaeta for the reduction
7		of carbon dioxide to methane. Energy & Environmental Science, 7, 408-415.
8	[33]	Schink, B. 1997. Energetics of syntrophic cooperation in methanogenic degradation.
9		Microbiology and Molecular Biology Reviews, 61, 262-280.
10	[34]	Shen, Y., Linville, J.L., Urgun-Demirtas, M., Schoene, R.P., Snyder, S.W. 2015.
11		Producing pipeline-quality biomethane via anaerobic digestion of sludge amended with
12		corn stover biochar with in-situ CO2 removal. Applied Energy, 158, 300-309.
13	[35]	Shrestha, P.M., Malvankar, N.S., Werner, J.J., Franks, A.E., Elena-Rotaru, A., Shrestha,
14		M., Liu, F.H., Nevin, K.P., Angenent, L.T., Lovley, D.R. 2014. Correlation between
15		microbial community and granule conductivity in anaerobic bioreactors for brewery
16		wastewater treatment. Bioresource Technology, 174, 306-310.
17	[36]	Stams, A.J.M., Plugge, C.M. 2009. Electron transfer in syntrophic communities of
18		anaerobic bacteria and archaea. Nature Reviews Microbiology, 7, 568-577.
19	[37]	Storck, T., Virdis, B., Batstone, D.J. 2016. Modelling extracellular limitations for
20		mediated versus direct interspecies electron transfer. The ISME journal, 10, 621-631.
21	[38]	Summers, Z.M., Fogarty, H.E., Leang, C., Franks, A.E., Malvankar, N.S., Lovley, D.R.
22		2010. Direct Exchange of Electrons Within Aggregates of an Evolved Syntrophic

1		Coculture of Anaerobic Bacteria. Science, 330 , 1413-1415.
2	[39]	Tian, T., Qiao, S., Li, X., Zhang, M., Zhou, J. 2017. Nano-graphene induced positive
3		effects on methanogenesis in anaerobic digestion. <i>Bioresource Technology</i> , 224 , 41-47.
4	[40]	Torrijos, M. 2016. State of Development of Biogas Production in Europe. Procedia
5		Environmental Sciences, 35 , 881-889.
6	[41]	Viggi, C.C., Rossetti, S., Fazi, S., Paiano, P., Majone, M., Aulenta, F. 2014. Magnetite
7		Particles Triggering a Faster and More Robust Syntrophic Pathway of Methanogenic
8		Propionate Degradation. Environmental Science & Technology, 48, 7536-7543.
9	[42]	Wei, X., Kusiak, A. 2012. Optimization of biogas production process in a wastewater
10		treatment plant. IIE Annual Conference. Proceedings. Institute of Industrial and Systems
11		Engineers (IISE). pp. 1.
12	[43]	Xia, A., Jacob, A., Herrmann, C., Tabassum, M.R., Murphy, J.D. 2015. Production of
13		hydrogen, ethanol and volatile fatty acids from the seaweed carbohydrate mannitol.
14		Bioresource Technology, 193 , 488-497.
15	[44]	Xia, A., Jacob, A., Tabassum, M.R., Herrmann, C., Murphy, J.D. 2016. Production of
16		hydrogen, ethanol and volatile fatty acids through co-fermentation of macro- and
17		micro-algae. Bioresource Technology, 205, 118-125.
18	[45]	Yamada, T., Sekiguchi, Y., Hanada, S., Imachi, H., Ohashi, A., Harada, H., Kamagata, Y.
19		2006. Anaerolinea thermolimosa sp. nov., Levilinea saccharolytica gen. nov., sp. nov. and
20		Leptolinea tardivitalis gen. nov., sp. nov., novel filamentous anaerobes, and description of
21		the new classes Anaerolineae classis nov. and Caldilineae classis nov. in the bacterial
22		phylum Chloroflexi. International Journal of Systematic and Evolutionary Microbiology,

56,	1331	-1340.
	56,	56 , 1331

2	[46]	Zhao, Z., Zhang, Y., Holmes, D.E., Dang, Y., Woodard, T.L., Nevin, K.P., Lovley, D.R.
3		2016a. Potential enhancement of direct interspecies electron transfer for syntrophic
4		metabolism of propionate and butyrate with biochar in up-flow anaerobic sludge blanket
5		reactors. Bioresour Technol, 209, 148-56.
6	[47]	Zhao, Z., Zhang, Y., Yu, Q., Dang, Y., Li, Y., Quan, X. 2016b. Communities stimulated
7		with ethanol to perform direct interspecies electron transfer for syntrophic metabolism of
8		propionate and butyrate. Water Res, 102, 475-84.
9	[48]	Zhao, Z.Q., Zhang, Y.B., Woodard, T.L., Nevin, K.P., Lovley, D.R. 2015. Enhancing
10		syntrophic metabolism in up-flow anaerobic sludge blanket reactors with conductive
11		carbon materials. Bioresource Technology, 191, 140-145.
12	[49]	Zhu, B., Xia, X., Xia, N., Zhang, S., Guo, X. 2014. Modification of fatty acids in
13		membranes of bacteria: implication for an adaptive mechanism to the toxicity of carbon
14		nanotubes. Environ. Sci. Technol., 48, 4086.
15		

1	List of figures and tables:
2	
3	Fig. 1 Effects of graphene and activated charcoal on biomethane yield and production rate from
4	ethanol: (a) biomethane yield, (b) biomethane production rate.
5	Fig. 2 Effects of graphene and activated charcoal on ethanol and acetate conversion: (a) ethanol
6	degradation kinetics, (b) ethanol degradation rate constant, (c) acetate degradation, and (d) overall
7	electron recovery.
8	Fig. 3 Mechanisms for extracellular cell-to-cell electron transfer in anaerobic digestion: (a)
9	mediated interspecies electron transfer, (b) direct interspecies electron transfer via graphene.
10	
11	Table1 Effects of graphene and activated charcoal on biomethane production kinetics.
12	Table 2 Three step reactions and thermodynamics in bioconversion of ethanol to biomethane.
13	Table 3 Bacterial community structures at genus level with/without graphene addition after
14	anaerobic digestion of ethanol. Genera with less than 1% abundances were classified into others.
15	Table 4 Archaeal community structures at genus level with/without graphene addition after
16	anaerobic digestion of ethanol. Genera with less than 1% abundances were classified into others.
17	



4

Fig. 1 Effects of graphene and activated charcoal on biomethane yield and production rate from
ethanol: (a) biomethane yield, (b) biomethane production rate. Results are the means and standard

deviations for duplicate experiments.

8

7



3

4 Fig. 2 Effects of graphene and activated charcoal on ethanol and acetate conversion: (a) ethanol 5 degradation kinetics, (b) ethanol degradation rate constant, (c) acetate degradation, and (d) overall 6 electron recovery. Results are the means and standard deviations for duplicate experiments.



MA

- 3 Fig. 3 Mechanisms for extracellular cell-to-cell electron transfer in anaerobic digestion: (a)
- 4 mediated interspecies electron transfer, (b) direct interspecies electron transfer via graphene.

5

6 Table1 Effects of graphene and activated charcoal on biomethane production kinetics.

Conductive materials	Biomethane yield	Peak biomethane	Kinetic model	l parameters			
concentration	(mL/g)	production rate (mL/g/d)	H_m (mL/g)	R_m (mL/g/d)	λ (d)	$T_m(\mathbf{d})$	R^2
No	556.1±53.3	80.1±0.2	579.7	86.9	0.89	3.34	0.9967
0.5 g/L graphene	670.9±16.0	83.0±1.1	711.2	99.1	0.81	3.45	0.9951
1.0 g/L graphene	695.0±9.1	95.7±7.6	718.4	116.2	0.60	2.87	0.9955
2.0 g/L graphene	662.9±14.7	84.6±1.3	695.7	102.8	0.70	3.19	0.9950
20.0 g/L activated charcoal	627.2±30.7	91.1±18.6	648.8	109.8	0.65	2.82	0.9939

7 Note: H_m , maximum methane yield potential; R_m , peak methane production rate; λ , lag-phase time; and T_m , peak time of methane fermentation.

31

8 Table 2 Three step reactions and thermodynamics in bioconversion of ethanol to biomethane.

Process	Reactions	$\Delta G_0'^a$ (kJ/mol)
1 Electron graducing sectoron	MIET: $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	9.68
1. Electron-producing acetogen	DIET: $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 5H^+ + 4e^-$	-149,64
2 Electron comming mothers con	MIET: $2H_2 + 1/2CO_2 \rightarrow 1/2CH_4 + H_2O$	-65.35
2. Electron-consuming methanogen	DIET: $4H^+ + 4e^- + 1/2CO_2 \rightarrow 1/2CH_4 + H_2O$	93.98
3. Acetate-consuming methanogen	$\rm CH_3\rm COO^- + \rm H^+ \rightarrow \rm CH_4 + \rm CO_2$	-35.91
Overall	$CH_{3}CH_{2}OH \rightarrow 3/2CH_{4} + 1/2CO_{2}$	-91.58

9 $^{a}\Delta G_{0}'$ is the free energy change of reaction under standard conditions at pH 7. Negative value indicates the reaction is thermodynamically favorable and proceeds

10 spontaneously.

Table 3 Bacterial community structures at genus level with/without graphene addition after anaerobic digestion of ethanol. Genera with less than 1% abundances

	Relative abundance in different anaerobic digestates (%)			
Genera	Inoculum	Digestate without graphene	Digestate with 1.0 g/L graphene	
Geobacter	0.29	8.43	9.94	
Pseudomonas	0.43	1.91	6.85	
Levilinea	7.64	11.59	6.2	
Clostridium	10.09	8.57	5.15	
Thermovirga	3.34	2.71	2.98	
Victivallis	0.38	2.89	2.73	
Aminobacterium	3.98	2.14	2.24	
Longilinea	0.85	2.71	2.22	
Desulfovibrio	0.05	2.27	1.96	
Synergistes	3.09	1.97	1.72	
Smithella	2.86	2.03	1.45	
Syntrophomonas	1.4	2.13	1.27	
Meniscus	1.86	1.69	1.24	
Bellilinea	1.27	1.54	0.9	
Others	42.92	34.04	39.39	
unclassified	19.55	13.38	13.76	
	G	33		

location into oth

Table 4 Archaeal community structures at genus level with/without graphene addition after anaerobic digestion of ethanol. Genera with less than 1% abundances 16

Relative abundance in diff	ferent anaerobic digestates (%)	
Inoculum	Digestate without graphene	Digestate with 1.0 g/L graphene
86.08	50.14	39.75
2.68	24.02	34.87
6.44	20.19	9.84
1.14	2.15	7.76
2.27	2.07	4.66
1.39	1.43	3.12
	Inoculum 86.08 2.68 6.44 1.14 2.27 1.39	Inoculum Digestate without graphene 86.08 50.14 2.68 24.02 6.44 20.19 1.14 2.15 2.27 2.07 1.39 1.43

lossified into oth 17

20 Graphical abstract



Graphene enhanced methane yield (+25%) and production rate (+20%) in AD of ethanol. 24

Microbial structures of electro-active bacteria and archaea were revealed after AD. 25

Direct interspecies electron transfer (DIET) via graphene was established in AD. 26

DIET sustained much higher electron transfer flux than hydrogen transfer. 27

28