

Title	Effects of foam nickel supplementation on anaerobic digestion: Direct interspecies electron transfer
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Publication date	2020-05-17
Original Citation	Guo, X., Sun, C., Lin, R., Xia, A., Huang, Y., Zhu, X., Show, P. L. and Murphy, J. D. (2020) 'Effects of foam nickel supplementation on anaerobic digestion: Direct interspecies electron transfer', Journal of Hazardous Materials, 399, 122830 (10 pp). doi: 10.1016/ j.jhazmat.2020.122830
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://www.sciencedirect.com/science/article/pii/ S0304389420308190 - 10.1016/j.jhazmat.2020.122830
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Download date	2025-04-15 15:50:51
Item downloaded from	https://hdl.handle.net/10468/10201



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Anaerobic digestion of wastewater



*Revised Manuscript

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20 Abstract

Stimulating direct interspecies electron transfer with conductive materials is a 21 22 promising strategy to overcome the limitation of electron transfer efficiency in syntrophic methanogenesis of industrial wastewater. This paper assessed the impact of 23 conductive foam nickel (FN) supplementation on the syntrophic methanogenesis and 24 found that addition of 2.45 g/L FN in anaerobic digestion increased the maximum 25 methane production rate by 27.4% (on day 3) while decreasing the peak production 26 time by 33% as compared to the control with no FN. Cumulative methane production 27 28 from day 2 to 6 was 14.5% higher with addition of 2.45 g/L FN than in the control. Levels of FN in excess of 2.45 g/L did not show benefits. Cyclic voltammetry results 29 30 indicated that the biofilm formed on the FN could generate electrons. The dominant 31 bacterial genera in suspended sludge were *Dechlorobacter* and *Rikenellaceae* DMER64, whereas that in the FN biofilm was Clostridium sensu stricto 11. The 32 dominant archaea Methanosaeta in the FN biofilm was enriched by 14.1% as 33 34 compared to the control. 35 Keywords: Direct interspecies electron transfer; Methane production; Industrial 36



wastewater; Biofilm; Biogas.

1. Introduction

40	In terms of world energy, the market for natural gas use is approximately twice
41	that of electricity. Anaerobic digestion (AD) may be viewed as a technology which
42	produces renewable methane and as such in the energy transition from fossil fuel to a
43	decarbonized future has a huge role to play in climate mitigation through reduction of
44	greenhouse gas emissions from combustion of gaseous fuels. AD is a well-understood
45	process but there are still areas where the technology may be improved [1]. Improving
46	the biological efficiency of AD, increasing the methane yields over shorter time
47	frames leads to improved resource, sustainability and cost.
48	Industrial wastewater such as dye wastewater and petroleum wastewater is
49	hazardous to aquatic organisms and induces potential environmental pollution due to
50	the presence of surfactants [2, 3]. AD is efficient and environmentally-friendly to
51	biodegrade industrial wastewater. Apart from the toxicity of the surfactants to
52	microbes, the limitation of electron transfer efficiency in syntrophic methanogenesis
53	blocks AD of industrial wastewater [2, 4]. In syntrophic methanogenesis, acetogens
54	can biodegrade volatile fatty acids (VFAs) and/or alcohols into acetic acid and H_2 .
55	However, a high H_2 partial pressure leads to a thermodynamically unfavorable
56	acetogenesis and restrains AD process [5]. Methanogens can consume H_2 to reduce
57	CO_2 for methane production, whilst maintaining a low H ₂ partial pressure (H ₂ < 10 ⁻⁴
58	atm) to facilitate acetogenesis [6]. This syntrophic methanogenesis between acetogens
59	and methanogens with H_2 as electron carriers is mediated interspecies electron
60	transfer (MIET) [7]. Direct interspecies electron transfer (DIET) characterized by the

61	electron transfer directly along the outer membrane c-type cytochrome (OmcS) and
62	pili of exoelectrogens was recently suggested to be an efficient alternative to MIET [6,
63	8]. This is attributed to the higher electron transfer rate without the diffusional
64	limitation of electron carriers and less energy consumption by eliminating synthesis of
65	electron carriers as compared to MIET [7]. As such stimulation of DIET is a
66	promising approach to boost electron transfer efficiency in AD of industrial
67	wastewater [9].
68	Conductive materials (CMs) were suggested to efficiently compensate OmcS and
69	pili for stimulatory of DIET [10, 11]. This is attributed to the fact that CMs can reduce
70	the energy consumption for synthesis of OmcS and pili, and its higher electrical
71	conductivity than pili (2-20 μ S·cm ⁻¹); examples include granular activated carbon and
72	polyaniline nanorods with electrical conductivities of 3000 μ S·cm ⁻¹ and 0.74 S·cm ⁻¹ ,
73	respectively [12]. Nevertheless, CMs with the properties of higher electrical
74	conductivity such as metallic CMs and feasibility to recovery and reuse is of great
75	appeal for application to stimulate DIET [10, 13].
76	Foam nickel (FN) functioning as a metallic CM exhibits much higher electrical
77	conductivities $(1.4 \times 10^7 \text{ S} \cdot \text{m}^{-1})$ than carbon based CMs and its three-dimensional
78	framework structure facilitates its recovery and reuse. The electrical conductivity of
79	nickel is higher than iron $(1.3 \times 10^6 \text{ S} \cdot \text{m}^{-1})$ and its ecotoxicity is much lower than
80	copper ion, [14]. The larger volume fraction of gas-filled pores of FN benefited the
81	development of electroactive biofilm when FN was employed as electrode in
82	microbial fuel cells (MFC) [15]. Given that these superior advantages of FN benefit

- ⁸³ for electron transfer, electroactive biofilm development, and feasibility for recovery
- ⁸⁴ and reuse, FN may be a promising CM to efficiently stimulate DIET and promote
- 85 syntrophic methanogenesis. The AD of nonionic surfactants in industrial wastewater
- ⁸⁶ is limited to the syntrophic methanogenesis of its important group linear alcohol
- 87 ethoxylates, where ethanol is the key intermediate and the stimulatory of its DIET can
- 88 boost the syntrophic methanogenesis [4]. The gap in the state of the art is that the
- 89 effects of FN supplementation on stimulatory of DIET and microbial community
- 90 composition shifts benefit for syntrophic methanogenesis have yet to be reported.
- 91 Moreover, FN is a core of reinforcing material in nickel-hydrogen batteries and is a
- 92 potential industrial waste after its discarded. As such, this study will provide a novel
- 93 approach to utilize discarded FN.
- In this study, the innovation is for the first time to calibrate the stimulatory of
- 95 DIET with metal foam (in the case of FN) for methane production improvement. This
- 96 can provide a novel approach to stimulate syntrophic methanogenesis. The objectives
- are to evaluate the influence of FN supplementation on biogas production, to
- 98 investigate the difference in microbial community composition between suspended
- ⁹⁹ sludge and biofilm foamed on FN, and to analyze the mechanism of DIET benefit for
- 100 methane production with FN supplementation.
- 101
- 102
- 103
- 104

105 **2. Materials and methods**

106 *2.1. Experimental materials*

107	The sludge was sourced from a rural household anaerobic digester in Chongqing,
108	stored for degassing for 90 days at room temperature in a plastic container, and
109	subsequently acclimatized on three separate occasions in our laboratory anaerobic
110	digester treating cellulose [16]. The total suspended sludge (TSS) of the inoculum was
111	77.2 g/L and the ratio of volatile suspended sludge (VSS) to TSS was 53.1%. The FN,
112	characterized by electrical conductivity of $1.4 \times 10^7 \text{ S} \cdot \text{m}^{-1}$, 97% porosity and a pore
113	size of 0.3 mm (Kunshan Guangjia New Material Ltd, China) was cut into blocks (10
114	\times 10 \times 5 mm, L \times W \times H) weighting 0.07 \pm 0.006 g each.

115

116 2.2. Batch experiments

Ethanol was selected as a model substrate to investigate the limitation of electron 117 transfer efficiency in syntrophic methanogenesis; it is also a simple feedstock so as 118 such considerations relating to recalcitrance are not of an issue and do not complicate 119 the findings. The assays of methane production potential were conducted in batch 120 experiments with 250 mL glass media bottle reactors. Each reactor was filled with 70 121 g inoculum, 1 mL absolute ethanol, and deionized water to make up the total working 122 volume of 200 mL. The initial ratio of inoculum to substrate in the reactors was 123 adjusted to 3.5:1; this ratio was chosen as ethanol is a readily degradable substrate in 124 AD [17]. The number of FN blocks supplemented in different treatments was 0, 7, 21, 125 35, and 70 with the corresponding concentration of FN added of 0, 2.45, 7.35, 12.25, 126

127	and 24.50 g/L; these are identified as control, FN 2.45, FN 7.35, FN 12.25, and FN
128	24.50, respectively. The blank of inoculum without ethanol added served as a negative
129	control. The pH in the reactors was initially adjusted to 7.5 using 6 M HCl and 6 M
130	NaOH solutions, and subsequently adjusted to 7.5 ± 0.1 every day. Initially the
131	reactors were purged with N_2 for 5 min and sealed to ensure an anaerobic condition.
132	Afterwards, the reactors were placed in a water bath to maintain the temperature of
133	the AD process at 35.0 \pm 0.1 $^{\circ}\mathrm{C}.$ Two mL of supernatant was sampled from each
134	reactor on day 0, 2, 4, 6, 8 and 10, and filtrated by 0.45 μ m filter membrane to assess
135	the concentration of ethanol and acetate during the AD process. The experimental
136	scheme is presented in Fig. 1.

- 137
- 138 2.3. Analytical methods

The produced biogas volume was assessed by a graduated gas container using the 139 water drainage method [18]. The concentration of methane was determined via a gas 140 chromatography system (GC; ThermoFisher, USA) equipped with a thermal 141 conductivity detector and a micro-packed column [19]. The concentration of VFAs 142 was analyzed with another GC system (Aglient 7890B, USA) equipped with a flame 143 ionization detector and a polar capillary column [18]. The concentration of nickel ion 144 in the suspension sampled on day 10 was assessed by inductively coupled 145 plasma-optical emission spectroscopy (ICP-OES, Optima 8000, USA) [20]. 146 147

148

149 2.4. Characteristics of biofilm adhered to FN

150	The FN carriers were collected from the assay FN 2.45 on day 10, rinsed with
151	phosphate-buffered saline (PBS) (pH 7.0-7.2) to remove the sludge, and subsequently
152	the biofilm adhered to the FN was investigated. The FN 2.45 carriers were then fixed
153	with 2.5% (m/v) glutaraldehyde in PBS solution, stepwise dehydrated with 10, 30, 50,
154	70, and 90% (v/v) ethanol, and coated with gold to facilitate imaging using scanning
155	electron microscopy (SEM) (Zeiss Auriga, Germany) [18].
156	The electrochemical activities of biofilm adhered to the FN were assessed with
157	cyclic voltammetry (CV) at a scan rate of $1.0 \text{ mV} \cdot \text{s}^{-1}$ and scan range from -0.4 to 0.1
158	V by using ethanol (40 mmol/L) and acetate (20 mmol/L) as electron donors,
159	respectively. The FN carriers obtained from the assay FN 2.45, platinum electrode and
160	Ag/AgCl electrode were used as anode, cathode, and reference electrode in a single
161	chamber MFC, respectively. The fresh FN was used as a control anode.
162	
163	2.5. Microbial community analysis
164	The sludge from the original inoculum, the control, and the FN suspension as well
165	as the biofilm adhered to the FN carriers in assay FN 2.45 after AD were sampled for
166	investigation of microbial community structure. The PowerSoil [@] DNA Isolation Kit
167	(MoBio Laboratories Inc., Carlsbad, CA, USA) was adopted to extract the genomic
168	DNA [21]. The primer sequences for 338F and 806R were used to amplify the V3-V4
169	region of bacterial 16S rRNA gene and listed as 5'-ACTCCTACGGGAGGCAGCA-3'
170	and 5'-GGACTACVSGGGTATCTAAT-3', respectively [22]. The primer sets of

171 Arch349F (5'-GYGCASCAGKCGMGAAW-3') and Arch806R

172	(5'-GGACTACVSGGGTATCTAAT-3') were used for amplification of the V3-V4
173	region of archaeal 16S rRNA gene [23]. Illumina HiSeq 2500 was used for 16S rRNA
174	gene sequencing. Raw sequences were assembled, screened, and trimmed. Sequences
175	obtained in the clone libraries were assigned different operational taxonomic units
176	(OTU) and each OTU represented 97% sequence identity. The neighbor-joining
177	phylogenetic trees were constructed to identify the representative OTUs along with
178	searching for the closest matched sequences with the BLAST program in the National
179	Center for Biotechnology Information (NCBI) database. The raw sequencing reads
180	were deposited in the NCBI SRA under project number PRJNA573494 with accession
181	SAMN12811488-SAMN12811490 and SAMN12816961-SAMN12816964.
182	

183 2.6. Data analysis

All the methane volumes presented in this study were normalized to standard
conditions (0 °C, 1 atm). The AD trials were carried out with three replicates, and the
results were expressed as mean ± standard deviation. Analysis of variance was
conducted to assess the significance of differences with Tukey test with GraphPad
Prism.

193 **3. Results and discussion**

212

194 *3.1. Influence of FN supplementation on methane production in AD process*

195 When ethanol was used as a model substrate in AD, the syntrophic methanogenesis of ethanol involves interspecies electron transfer. In this AD system, 196 the hydrogen produced was hard to detect due to the high activities of 197 hydrogenotrophic methanogens. The methane production rate (MPR) and specific 198 methane yield (SMY) in the control are depicted in Figs. 2a and 2b, respectively. As 199 the cumulative duration of the batch experiment increased to 2 days, the MPR rapidly 200 201 increased to 66.8 mL/g-ethanol/d, and the SMY increased to 110.7 mL/g-ethanol. Almost no lag phase time was observed due to the high inoculum to substrate ratio of 202 3.5 (Fig. 2b). As the time increased to day 4.5, the MPR quickly increased and peaked 203 at 91.5 mL/g-ethanol/d (Fig. 2a). Given that ethanol was readily degraded into acetate, 204 the accumulation of acetate not only contributed to higher MPR, but also led to a 205 significant drop in pH (Fig. 2c). Methanogens are considered to be sensitive to pH 206 207 change with the optimum pH range from 6.7 to 7.8 [24]. Therefore, the lower pH inhibited methanogenic activity leading to a reduction in potential scale of MPR (Fig. 208 $\frac{2a}{2a}$). As further time past (from day 6 to 8 d) the MPR quickly decreased and by day 8 209 methane production was minimal owing to the depletion of acetate. Maximum SMY 210 of 500.0 mL/g-ethanol was obtained on day 10 (Fig. 2b). 211

trends of SMY and MPR were in line with the control during the initial two days (Figs.

When the FN was supplemented at a concentration of 2.45 g/L (FN 2.45), the

214 2a and 2b). As the time of experiment increased from day 2 to 3, the MPR quickly

215	increased to the peak of 94.5 mL/g-ethanol/d, 27.4% higher than the control ($p < $
216	0.01). The time to achieve maximum MPR reduced by 33% as compared to that of the
217	control (4.5 d) (Fig. $\frac{2}{2a}$). From day 3 to 4.5, the MPR was almost stable regardless of a
218	lower pH in the reactor, suggesting a high performance with this level of FN
219	supplementation (Fig. $\frac{2}{2c}$). From day 2 to 6 the cumulative methane production was
220	14.5% higher with FN 2.45 than in the control ($p < 0.01$, Fig. 2a). It is postulated that
221	the enhancement of SMY and MPR may be attributed to the stimulation of DIET
222	induced by FN. Afterwards, the MPR quickly dropped to near zero on day 8 due to the
223	depletion of acetate. Finally, the SMY reached 514.8 mL/g-ethanol on day 10,
224	corresponding to 70.5% of the theoretical value (Fig. 2b). No substantial enhancement
225	on SMY with stimulatory of DIET was observed as compared to the control, which is
226	consistent with previous findings [25]. This may be related to the ready
227	biodegradability of ethanol and the high activities of inoculum.
228	When the FN concentration was increased from 7.35 to 24.50 g/L, the maximum
229	MPR reduced from 63.7 to 3.7 mL/g-ethanol/d (Fig. $2a$), and SMY decreased from
230	384.2 to 24.0 mL/g-ethanol (Fig. $\frac{2}{2b}$). The inhibition is attributed to the increased
231	dissolution of nickel ion (4.0-4.7 mg/L) in the solution (Fig. 2d). This was consistent
232	with the previous find that overloading of nickel ions suppressed microbial activity
233	and led to the reduction in SMY and MPR [26]. Similar trends of inhibition induced
234	by higher dose of CMs supplementation such as nano-graphene, polyaniline nanorods,
235	and graphene were documented by others [12, 16].
226	

3.2. Influence of FN supplementation on concentration of ethanol and acetate in AD
process

The concentrations of ethanol and acetate in the AD process are shown in Fig $\frac{3}{2}$. 239 For the first 4 days the ethanol in both the control and FN 2.45 groups was rapidly 240 degraded (Figs. 3a and 3b). At the same time acetate in the control and the FN 2.45 241 groups increased gradually and the FN 2.45 group peaked at about 37.0 mmol/L on 242 day 4. The accumulation of acetate was consistent with the significant drop in pH (Fig. 243 $\frac{3c}{3c}$). Although the trends for ethanol and acetate in the control and FN 2.45 were 244 245 similar, the acetate produced with the FN 2.45 assay was slightly higher than the control, thus contributing to a higher MPR in FN 2.45 (Fig. 2a). The enhancement of 246 acetate production with FN 2.45 can be ascribed to the efficient stimulation of DIET 247 248 by CMs [27, 28]. From day 4 to 6 the ethanol in the control and FN 2.45 was depleted to minimal levels, while the concentration of acetate gradually decreased due to the 249 consumption of acetate by acetotrophic methanogens for methane production. From 250 day 6 to 10 the acetate in the control and FN 2.45 was depleted, whilst beyond day 7 251 minimal amounts of methane were produced (Figs. 3a and 3b). 252 When the FN concentration was increased from 7.35 to 24.50 g/L, acetogenesis 253

was significantly inhibited (Figs. <mark>3c, 3d, and 3e</mark>). Lower levels of inhibition were

apparent with FN 7.35 than that with FN 12.25 and FN 24.50. Ethanol was depleted

on day 6 with FN 7.35, whereas for FN 12.25 and FN 24.50 the ethanol concentration

was still at a high concentration on day 10. Acetate production peaked on day 4 with

FN 7.35 and its consumption was low by day 10. Almost no acetate accumulated with

259	FN 12.25 and FN 24.50. The degradation of ethanol and acetate was significantly
260	inhibited with FN 12.25 and FN 24.50. This is ascribed to the toxicity of higher
261	concentration of nickel ions to microbes (Fig. 2d).
262	
263	3.3 Influence of FN supplementation on microbial community in AD process
264	3.3.1 Microbial community in suspended sludge
265	The original inoculum, suspension from the control, and suspension from optimal
266	FN supplementation group (FN 2.45) termed as FN 2.45 suspension, were collected
267	for investigating microbial community in suspended sludge. Alpha diversity indexes
268	for estimating the microbial richness (i.e. ACE and Chao) and microbial evenness (i.e.
269	Shannon and Simpson) are presented in Table 1. The bacterial richness and evenness
270	in the FN 2.45 suspension were lower than in the control indicating that FN 2.45
271	supplementation significantly influenced bacterial community. The dominant bacteria
272	in the inoculum and control were <i>Rikenellaceae</i> DMER64. In the FN 2.45 suspension
273	<i>Rikenellaceae</i> DMER64 and <i>Dechlorobacter</i> dominated (Fig. 4a). The relative
274	abundance of <i>Rikenellaceae</i> DMER64 in the inoculum, the control, and the FN 2.45
275	suspension were 23.3%, 11.7%, and 18.7%, respectively, which were higher than in
276	the biofilm adhered to the FN 2.45 carriers (3.2%) (Fig. 4a). <i>Rikenellaceae</i> DMER64
277	is an active member of the bacterial community in common AD systems [29], which
278	may be beneficial for interspecies hydrogen transfer. The relative abundance of
279	Dechlorobacter in the FN 2.45 suspension was significantly enriched as compared to
280	the control and the inoculum (Fig. 4b). <i>Dechlorobacter</i> is a potential electroactive

281	genus as it enables degradation of benzene and xylene with oxygen, nitrate, and
282	chlorate as the electron acceptor [30]. The Dechlorobacter_OTU2 in the FN 2.45
283	suspension was closely affiliated to FN436157_s (99.3% sequence identity) (Fig. $\frac{1}{4c}$).
284	FN436157_s was a dominant denitrifier in hollow fiber-membrane biofilm reactors
285	for treating high-strength nitrogen wastewater [31]. DIET is also involved in
286	denitrification [32]. Therefore, <i>Dechlorobacter_</i> OTU2 may be a potential
287	exoelectrogen in the FN 2.45 suspension.
288	The archaeal richness and evenness in the control and the FN 2.45 suspension
289	were identical (Table $\frac{1}{1}$). The dominant archaea in the inoculum, the control, and the
290	FN 2.45 suspension were Methanosaeta, Methanospirillum, and Methanolinea (Fig.
291	$\frac{5}{5a}$). The relative abundance of <i>Methanosaeta</i> in the control and the FN 2.45
292	suspension decreased to 17.8% and 20%, respectively, as compared to the inoculum.
293	This was due to the enrichment of Methanospirillum in the control and FN 2.45

suspension. The abundance of Methanospirillum in the control and FN 2.45 294

suspension increased between 4.8 and 9.5 times by comparison to the original 295

inoculum (Fig. 5b). The relative abundance of hydrogenotrophic methanogens, 296

Methanospirillum and Methanolinea, were influenced with FN supplementation, 297

which is in a similar pattern to previous studies [28, 33]. The enrichment of 298

Methanospirillum in the FN 2.45 suspension indicates its potential roles in DIET. 299

Although the archaellum of *Methanospirillum hungatei* was electrically conductive 300

[34], the actual role of Methanospirillum in DIET remains elusive as co-cultures of 301

Methanospirillum and exoelectrogen via DIET have not been validated. 302

304	The electrochemical activity of biofilm adhered to the FN 2.45 carriers was
305	assessed by CV analysis (Fig. $\frac{6}{6a}$). As ethanol and acetate acted as electron donors in
306	the DIET, ethanol (40 mmol/L) and acetate (20 mmol/L) were used in a MFC to
307	assess the electrochemical properties of the biofilm, respectively. Two distinct
308	oxidation peaks at potential of -0.15 V and -0.25 V (versus Ag/AgCl) were observed
309	(Fig. $\frac{1}{6a}$), which were in the range of -0.45 V to -0.1 V belonging to the bio-oxidizing
310	volatile organic acid by exoelectrogens [35]. The peak currents produced by biofilm
311	adhered to the FN 2.45 carriers with ethanol and acetate as electron donors were 8 mA
312	and 10 mA, respectively (Fig. $\frac{6}{6a}$). The rapid drop of current at -0.15 V may be
313	ascribed to the inhibition of metabolite (i.e. H^+) to exoelectrogens. In contrast, current
314	was hardly detected with the FN control as anode (Fig. $\frac{1}{6b}$). It is suggested the biofilm
315	adhered to FN 2.45 carriers is capable of utilizing ethanol and acetate as electron
316	donors to produce electrons. The similar finding was demonstrated that carbon cloth
317	supplementation facilitated the electroactive biofilm development [36]. Therefore, the
318	electroactive biofilm adhered to the FN 2.45 carriers could facilitate electron transfer
319	between syntrophic consortia, thus enhancing the MPR with FN 2.45 (Fig. $\frac{1}{2a}$).

303 3.3.2 Electrochemical characteristics of biofilm adhered to FN carriers

320

321 3.3.3. Microbial community in the biofilm adhered to FN carriers

The biofilm adhered to FN 2.45 carriers was sonicated and collected (denoted as FN 2.45 biofilm) to investigate the microbial community in the biofilm. The bacterial evenness in the FN 2.45 biofilm was significantly lower than in the control probably

325	due to the preferences of FN 2.45 to exoelectrogens (Table $\frac{1}{1}$). The dominant bacterial
326	genera in the FN 2.45 biofilm were Clostridium sensu stricto, Acinetobacter, and
327	Dechlorobacter (Fig. 4a). The Clostridium sensu stricto in the FN 2.45 biofilm
328	accounted for 29.6% relative abundance, which is significantly higher than in the
329	control and FN 2.45 suspension (Figs. 4a and 4b). The <i>Clostridium sensu stricto</i> was
330	also significantly enriched when conductive polyaniline nanorods was added to
331	stimulate DIET [12]. Clostridium, a genus of gram-positive bacteria, was shown to be
332	electrochemically active [37, 38]. The enriched Clostridium sensu stricto accordingly
333	may facilitate DIET with methanogens in the FN 2.45 biofilm. Clostridium sensu
334	stricto 11 accounting for 19.7% relative abundance was enriched significantly as
335	compared to that in the inoculum, the control and the FN 2.45 suspension (Fig. $\frac{4b}{4b}$).
336	Clostridium sensu stricto 11_OTU3 was closely affiliated to Clostridium guangxiense
337	strain xsk1 and Clostridium guangxiense strain ZGM211 (100% sequence identity)
338	(Fig. 4c). Clostridium guangxiense ZGM211 is a hydrogen-producing strain [39, 40]
339	and might shift its metabolic process from producing H_2 to H^+ and electrons when
340	conductive FN 2.45 is present. The remarkable enrichment on Clostridium sensu
341	stricto 11_OTU3 in FN 2.45 biofilm implied it played a vital role in DIET.
342	Acinetobacter_OTU8, Acinetobacter_OTU373 and Acinetobacter_OTU653 were
343	related to Acinetobacter sp. strain 18H6A8 (Fig. $\frac{4}{4c}$). Acinetobacter was reported as
344	an electroactive genus in Mn oxidizing/reducing and is closely related to the
345	extracellular electron transfer process [41, 42]. Therefore, the significant enrichment
346	of <i>Acinetobacter</i> in the FN 2.45 biofilm (i.e. 4.2 times higher compared to the control)

347 revealed its potential contribution to DIET in the AD process.

348	The archaeal evenness in the FN 2.45 biofilm was lower than in the control
349	probably due to more electrotrophic methanogens in the biofilm than in the
350	suspension. The dominant archaea in the FN 2.45 biofilm were Methanosaeta,
351	Methanospirillum, and Methanolinea (Fig. 5a). The abundance of Methanosaeta in
352	the FN 2.45 biofilm increased by 75.8% as compared to the original inoculum.
353	Compared to the control, the relative abundance of Methanosaeta in the FN 2.45
354	biofilm was enriched by 14.1%. Methanosaeta harundinacea (JCM-13211) was
355	reported to enable DIET with Geobacter metallireducens [6]. The same pattern that
356	Methanosaeta was enriched with CMs supplementation to stimulate DIET was also
357	reported [43, 44]. The enhancement of <i>Methanosaeta</i> in response to CMs
358	supplementation revealed the stimulatory of DIET in the AD process. Nevertheless,
359	decrease in Methanosaeta with CMs supplementation has also been documented [16].
360	The variation in these findings is ascribed to the difference of feedstocks and rector
361	configuration. Methanosaeta_OTU1 was closely affiliated to Methanosaeta concilii
362	GP-6 (100% sequence identity) and Methanosaeta harundinacea (JCM-13211) (Fig.
363	5c). Methanosaeta_OTU64 was affiliated to Methanonethylovorans hollandica DSM
364	15978 (98.4% sequence identity) and <i>Methanosarcina barkeria</i> DSM800 (Fig. 5c).
365	Hence, the enhancement on <i>Methanosaeta</i> in the FN 2.45 biofilm as compared to the
366	control implied that <i>Methanosaeta</i> enable to conduct DIET with exoelcetrogens.
367	

368

3.4 Integrated interspecies electron transfer with FN supplementation

370	The syntrophic consortia involved in interspecies electron transfer with FN 2.45
371	supplementation is shown in Fig. 7. The enrichment on syntrophic consortia such as
372	Geobacter, Methanosarcina, and Methanosaeta with CMs supplementation indicated
373	the stimulatory of DIET [45, 46]. However, the syntrophic consortia capable of direct
374	interspecies electron transfer are not limited to the aforementioned genera.
375	Based on the microbial community shifts in the control, the FN 2.45 biofilm, and
376	the FN 2.45 suspension (Fig. $\frac{1}{7}$) as well as the functions reported in previous works,
377	the potential syntrophic consortia capable of DIET and MIET are discussed. The
378	potential hydrogen-producing bacteria for MIET was Rikenellaceae DMER 64 due to
379	its relative abundance significantly higher than in the FN 2.45 biofilm. Rikenellaceae
380	DMER 64 transferred the electron carrier H_2 to hydrogenotrophic methanogens (i.e.
381	Methanospirillum and Methanolinea). Both the relative abundance of
382	Methanospirillum in the FN 2.45 suspension and Methanolinea in the control were
383	higher than those in the FN 2.45 biofilm, suggesting that MIET played an important
384	role in the suspension. The potential exoelectrogens in DIET were Acinetobacter,
385	Clostridium sensu stricto 11 and Dechlorobacter due to their enrichment with FN
386	2.45 supplementation. Acinetobacter and Clostridium sensu stricto 11 were the
387	predominant exoelectrogens in the FN 2.45 biofilm. Dechlorobacter were the
388	predominant exoelectrogens in the FN 2.45 suspension. These potential
389	exoelectrogens produced electrons in acetogenesis and transferred it to Methanosaeta,
390	which reduced CO ₂ with electrons to produce methane and metabolized acetate to

391	methane as well. The relative abundance of exoelectrogens in the FN 2.45 biofilm
392	was higher than that in the control, indicating DIET played an important role in the
393	FN 2.45 biofilm and MIET was dominant in the control. The higher abundance of
394	Methanosaeta also indicated DIET dominance in the FN 2.45 biofilm.
395	DIET and MIET generally coexist in the AD process, whereas their contribution
396	ratio to interspecies electron transfer is influenced by not only the characteristics of
397	CMs, but also the fermentation conditions including microbial activity, substrate
398	biodegradability, and rector configuration [47, 48]. Previous studies suggested DIET
399	predominated in up-flow anaerobic sludge blanket reactors due to the sludge bed
400	facilitating syntrophic consortia to form biofilm [6]. Moreover, interspecies electron
401	transfer in the AD system with conductive carbon cloth supplementation was
402	postulated to shift from interspecies hydrogen transfer to DIET [49]. The relative
403	abundance of Geobacter attached on conductive carbon cloth was more than that in
404	suspended sludge [49]. Therefore, it is postulated that biofilm is more favorable for
405	syntrophic consortia to perform DIET than suspended sludge. This is explained in two
406	ways: 1) biofilm keeps consortia tightly in contact and decreases the distance for
407	electron transfer; 2) the conductive matrix of biofilm facilitates interspecies electron
408	transfer [50]. This was in accordance with the evidence that more exoelectrogens and
409	electrotrophic methanogens were present in the FN 2.45 biofilm as compared to that
410	in the control. Therefore, biofilm adhered to CMs is also a favorable source to
411	investigate the syntrophic consortia. Apart from FN 2.45 facilitating formation of
412	conductive biofilm, the FN 2.45 may also compensate OmcS and pili in transfer of

- electrons. FN 2.45 supplementation, therefore, improves the efficiency of energy
- 414 utilization and methane production via reducing energy consumption for synthesis of
- 415 OmcS and pili.

416	A comparative study about the stimulatory of methanogenesis by CMs is depicted
417	in Table 2. Various carbon-based or metal-based CMs have been calibrated and the
418	methane production improvements were ascribed to the stimulatory of DIET by CMs.
419	The direct evidence of DIET is the defined coculture assay, where DIET is the sole
420	approach for electron transfer. However, such coculture is limited due to that the
421	cultured microbes in the habitat like anaerobic sludge are minor. Consequently,
422	integrated evidence including the increase in MPR and/or SMY, characteristics of
423	electroactive biofilm, and identification of DIET-partners is required to validate the
424	stimulatory of DIET [51]. In this study, the increase in maximum MPR by 27.4% is
425	ascribed to the stimulatory of DIET by FN 2.45 supplementation, which is consistent
426	with the development of electroactive biofilm on FN and the enrichment of
427	DIET-partners benefit for DIET.

- 428
- 429 **3.5** *Trends and Perspectives*
- 430 Resource utilization of industrial wastewater to reduce its hazardous to ecological
- 431 environment has attracted worldwide attention [2]. For example, numbers of policies
- and lows have been implemented to regulate and improve wastewater quality in China.
- 433 The State Council of the People's Republic of China issued the "Action Plan" calling
- 434 for treatment of water pollution in concentrated industrial areas on a centralized basis

- 435 [52]. The Ministry of Ecology and Environment of the People's Republic of China set
- 436 the national wastewater discharge standard for 12 different industrial sectors [53]. AD
- 437 can efficiently degrade the organic matter in industrial wastewater for biogas
- 438 generation and wastewater treatment. In 2019, the National Development and Reform
- 439 Commission set a target of annual production of bio-natural gas exceeding 10 billion
- 440 cubic meters by 2025 [54]. Advanced technologies to accelerate AD of wastewater are
- desired. This study showed the FN, which may be derived from battery industry, can
- 442 efficiently boost AD performance in terms of an increase in specific methane yield by
- 443 11.2% on day 6 and the maximum methane production rate by 27.4. Allowing a 10%
- 444 enhancement in methane yield during AD of dye wastewater with an HRT of 6 d at
- 445 FN concentration of 2.45 kg/m³, only 1.4 years is expected to recover the investment
- 446 of supplemented FN due to the extra methane production.
- 447 To demonstrate the enhancement effect of FN during the AD of real industrial
- 448 wastewater in full-scale, a long-term continuous experiment using dye wastewater or
- 449 papermaking wastewater in an upflow anaerobic sludge blanket reactor is necessary to
- 450 fully assess the impacts of FN supplementation on methane production. Moreover, the
- 451 modification of the surface properties of FN such as an increase in biocompatibility or
- 452 roughness to enhance biofilm formation may be beneficial for further stimulatory of
- 453 **DIET.**
- 454
- 455
- 456

457 **4. Conclusion**.

458	When FN was supplemented at a concentration of 2.45 g/L, a 27.4% increase in
459	maximum MPR was observed on day 3; the time to maximum MPR reduced by 33%
460	as compared to the no FN group. The electroactive biofilm formed on FN 2.45
461	carriers (FN 2.45 biofilm) could generate electrons. The potential exoelectrogens
462	Clostridium sensu stricto and dominant methanogen Methanosaeta were enriched
463	significantly benefit for DIET in the FN 2.45 biofilm. FN supplementation would
464	have a significant potential to stimulate DIET and accelerate the syntrophic
465	methanogenesis during the AD of industrial wastewater.
466	
467	Acknowledgements
468	This work was supported by the National Natural Science Foundation of China
469	(No. 51876016), the State Key Program of National Natural Science of China (No.
470	51836001), the Venture & Innovation Support Program for Chongqing Overseas
471	Returnees (No. cx2019040), and the Young Elite Scientists Sponsorship Program by
472	CAST (2018QNRC001). The collaborative work was also supported by Science
473	Foundation Ireland (SFI) through the MaREI centre for energy, climate and marine
474	under Grant No. 12/RC/2302 and 16/SP/3829. Dr Richen Lin acknowledges the
475	support from the European Union's Horizon 2020 research and innovation programme
476	under the Marie Skłodowska-Curie grant (No. 797259) and the Environmental

477 Protection Agency - Ireland (2018-RE-MS-13).

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List of abbreviations

AD	anaerobic digestion	MPR	methane production rate
CMs	conductive materials	MFC	microbial fuel cells
CV	cyclic voltammetry	NCBI	National Center for
			Biotechnology Information
DIET	direct interspecies electron	OmcS	outer membrane c-type
	transfer		cytochrome
FN	foam nickel	OTU	operational taxonomic units
FN 2.45	the group with FN added at	PBS	phosphate-buffered saline
	the concentration of 2.45 g/L		
FN 2.45	biofilm adhered to FN 2.45	SEM	scanning electron
biofilm	carriers		microscopy
FN 2.45	suspension from optimal FN	SMY	specific methane yield
suspension	supplementation group		
GC	gas chromatography system	TSS	total suspended sludge
ICP-OES	inductively coupled	VFAs	volatile fatty acids
	plasma-optical emission		
	spectroscopy		
MIET	mediated interspecies electron	VSS	volatile suspended sludge
	transfer		

634	List of	tables	and	figures:
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Table 1 Richness and diversity indexes of microbial community after AD

- **Table 2** Summary of studies about the stimulatory of methanogenesis by CMs
- 638

635

639	Fig. 1 The ex	perimental	scheme o	of stimulator	y of DIET	with FN	supplementa	tion
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- **Fig. 2** Influence of FN supplementation on (a) methane production rate, (b) specific
- 641 methane yield, (c) pH changes, (d) concentration of nickel ion after AD.

Fig. 3 Influence of FN supplementation on the concentrations of ethanol and acetate

in different treatments (a) control, (b) FN 2.45, (c) FN 7.35, (d) FN 12.25, (e) FN
24.50.

Fig. 4 (a) Bacterial community structure at genus level after AD, (b) Log₂ fold change
of abundance of dominant genera in the control, FN 2.45 biofilm, and FN 2.45
suspension to inoculum, (c) Neighbor-joining phylogenetic tree of representative
species.

Fig. 5 (a) Archaeal community structure at genus level after AD, (b) Log₂ fold change
of abundance of dominant genera in the control, FN 2.45 biofilm, and FN 2.45
suspension to inoculum, (c) Neighbor-joining phylogenetic tree of representative
species.

- **Fig. 6** Cyclic voltammogram measured in the single chamber MFC. (a) FN 2.45
- carriers as anode, (b) FN control as anode. Insets for a and b show SEM images of FN

655 2.45 carriers and FN control, respectively.

Fig. 7 Syntrophic consortia involved in interspecies electron transfer.

_	Sample	Ace	Chao	Shannon	Simpson	Coverage
Bacteria	Control	612	617	4.60	0.0257	1.0
	FN ^a 2.45 suspension	596	598	3.99	0.0696	1.0
	FN 2.45 biofilm	611	618	3.97	0.0596	1.0
	Inoculum	601	613	4.04	0.0552	1.0
Archaea	Control	43	43	1.61	0.3916	1.0
	FN 2.45 suspension	43	43	1.55	0.3871	1.0
	FN 2.45 biofilm	45	44	1.34	0.4972	1.0
	Inoculum	45	45	1.28	0.5562	1.0

657 Table 1 Richness and diversity indexes of microbial community after AD

658 ^a Foam nickel

Substrate	CM ^a	MPR ^b	Electroactive biofilm	Potential DIET-partners	Refs
Azo dye (RR2 ^c)	Ferroferric	+	NA^d	Paludibacter and	[2]
Phenol	Biochar	+	NA	Methanosarcina Geobacter and Methanosaeta	[11]
WAS ^e	$ZVI^{\rm f}+Fe_3O_4$	+	NA	Syntrophomonas and	[27]
				Methanosaeta	
Glycine	Graphene	+	NA	Sedimentibacter and	[16]
				Methanobacterium	
Ethanol	Graphene	+	NA	Geobacter and	[28]
				Methanobacterium	
Sucrose	Polyaniline	+	NA	Clostridium sensu stricto and	[12]
	nanorods			Methanosaeta	
Sodium lactate	Stainless steel	+	+	NA	[35]
Ethanol	Foam nickel	+	+	Clostridium sensu stricto and	This
				Methanosaeta	study

660 **Table 2** Summary of studies about the stimulatory of methanogenesis by CMs

661 ^a conductive material; ^b Methane production rate; ^c Reactive Red 2; ^d Not available; ^e Waste

activated sludge; ^f Zero-valent iron; + significant increase.



Fig. 1 The experimental scheme of stimulatory of DIET with FN supplementation.
MPR and SMY denote methane production rate and specific methane yield,
respectively.



Fig. 2 Influence of FN supplementation on (a) methane production rate, (b) specific methane yield, (c) pH changes, (d) concentration of nickel ion after AD. Each value is an average of triplicate assays, and each bar indicates \pm standard deviation (n=3).



Fig. 3 Influence of FN supplementation on the concentrations of ethanol and acetate in different treatments (a) control, (b) FN 2.45, (c) FN 7.35, (d) FN 12.25, (e) FN 24.50. Each value is an average of triplicate assays, and each bar indicates \pm standard deviation (n=3).

685 (a)



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Fig. 4 (a) Bacterial community structure at genus level after AD, (b) Log_2 fold change of abundance of dominant genera in the control, FN 2.45 biofilm, and FN 2.45 suspension to inoculum, (c) Neighbor-joining phylogenetic tree of representative species.



702 Fig. 5 (a) Archaeal community structure at genus level after AD, (b) Log₂ fold change 703 of abundance of dominant genera in the control, FN 2.45 biofilm, and FN 2.45 suspension to inoculum, (c) Neighbor-joining phylogenetic tree of representative 704 705 species.

706 (a)





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Fig. 6 Cyclic voltammogram measured in the single chamber MFC. (a) FN 2.45

carriers as anode, (b) FN control as anode. Insets for a and b show SEM images of FN

712 2.45 carriers and FN control, respectively.



Fig. 7 Syntrophic consortia involved in interspecies electron transfer.

Stimulating direct interspecies electron transfer with conductive materials is a promising strategy to overcome the limitation of electron transfer efficiency in syntrophic methanogenesis of industrial wastewater. This paper assessed the impact of conductive foam nickel (FN) supplementation on the syntrophic methanogenesis and found that addition of 2.45 g/L FN in anaerobic digestion increased the maximum methane production rate by 27.4% (on day 3) while decreasing the peak production time by 33% as compared to the control with no FN. Cumulative methane production from day 2 to 6 was 14.5% higher with addition of 2.45 g/L FN than in the control. Levels of FN in excess of 2.45 g/L did not show benefits. Cyclic voltammetry results indicated that the biofilm formed on the FN could generate electrons. The dominant bacterial genera in suspended sludge were *Dechlorobacter* and *Rikenellaceae* DMER64, whereas that in the FN biofilm was *Clostridium sensu stricto* 11. The dominant archaea *Methanosaeta* in the FN biofilm was enriched by 14.1% as compared to the control.

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