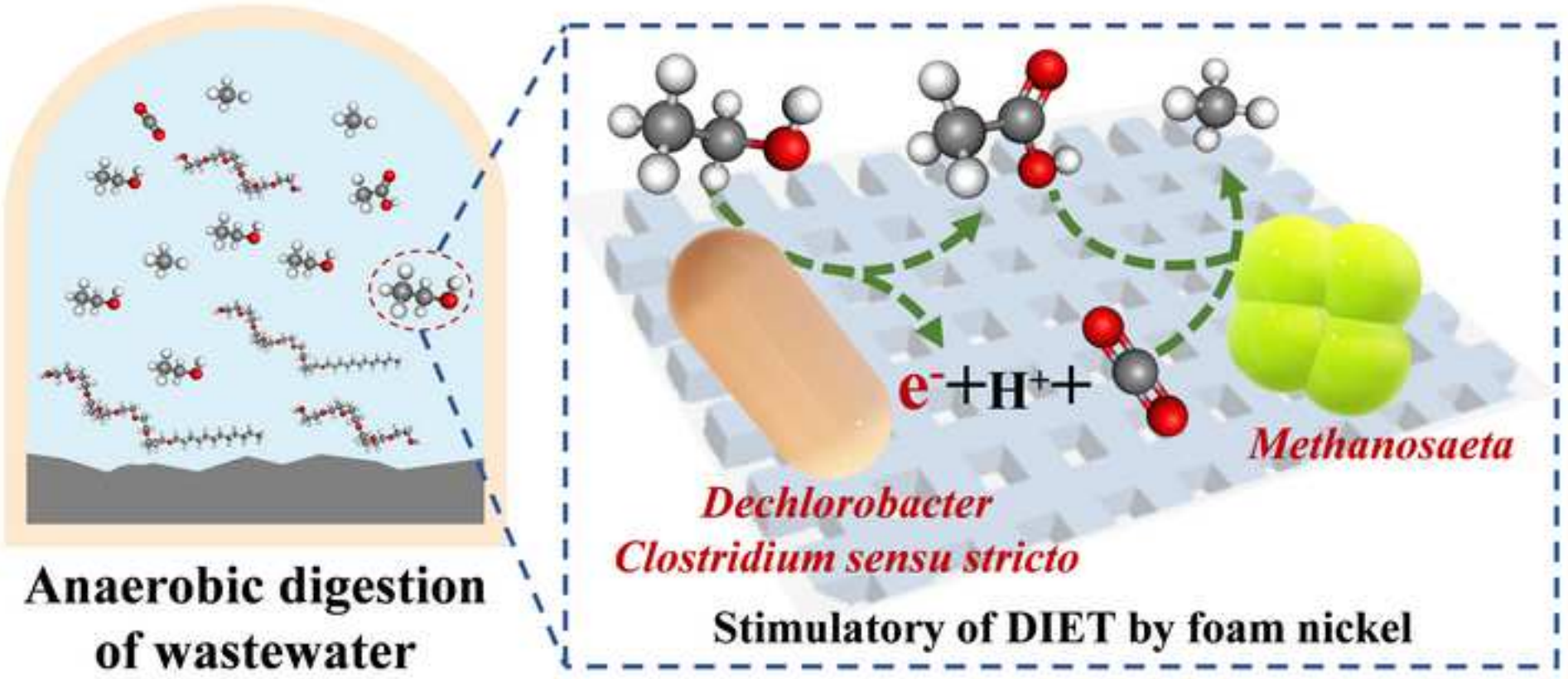
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*Graphical Abstract



# Effects of foam nickel supplementation on anaerobic digestion: direct interspecies electron transfer

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## Abstract

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.



**Keywords:** Direct interspecies electron transfer; Methane production; Industrial wastewater; Biofilm; Biogas.

## 1. Introduction

In terms of world energy, the market for natural gas use is approximately twice that of electricity. Anaerobic digestion (AD) may be viewed as a technology which produces renewable methane and as such in the energy transition from fossil fuel to a decarbonized future has a huge role to play in climate mitigation through reduction of greenhouse gas emissions from combustion of gaseous fuels. AD is a well-understood process but there are still areas where the technology may be improved [1]. Improving the biological efficiency of AD, increasing the methane yields over shorter time frames leads to improved resource, sustainability and cost.

Industrial wastewater such as dye wastewater and petroleum wastewater is hazardous to aquatic organisms and induces potential environmental pollution due to the presence of surfactants [2, 3]. AD is efficient and environmentally-friendly to biodegrade industrial wastewater. Apart from the toxicity of the surfactants to microbes, the limitation of electron transfer efficiency in syntrophic methanogenesis blocks AD of industrial wastewater [2, 4]. In syntrophic methanogenesis, acetogens can biodegrade volatile fatty acids (VFAs) and/or alcohols into acetic acid and $H_2$. However, a high $H_2$ partial pressure leads to a thermodynamically unfavorable acetogenesis and restrains AD process [5]. Methanogens can consume $H_2$ to reduce $CO_2$ for methane production, whilst maintaining a low $H_2$ partial pressure ($H_2 < 10^{-4}$ atm) to facilitate acetogenesis [6]. This syntrophic methanogenesis between acetogens and methanogens with $H_2$ as electron carriers is mediated interspecies electron transfer (MIET) [7]. Direct interspecies electron transfer (DIET) characterized by the

electron transfer directly along the outer membrane c-type cytochrome (OmcS) and pili of exoelectrogens was recently suggested to be an efficient alternative to MIET [6, 8]. This is attributed to the higher electron transfer rate without the diffusional limitation of electron carriers and less energy consumption by eliminating synthesis of electron carriers as compared to MIET [7]. As such stimulation of DIET is a promising approach to boost electron transfer efficiency in AD of industrial wastewater [9].

Conductive materials (CMs) were suggested to efficiently compensate OmcS and pili for stimulatory of DIET [10, 11]. This is attributed to the fact that CMs can reduce the energy consumption for synthesis of OmcS and pili, and its higher electrical conductivity than pili (2-20 $\mu S \cdot cm^{-1}$); examples include granular activated carbon and polyaniline nanorods with electrical conductivities of 3000 $\mu S \cdot cm^{-1}$ and 0.74 $S \cdot cm^{-1}$, respectively [12]. Nevertheless, CMs with the properties of higher electrical conductivity such as metallic CMs and feasibility to recovery and reuse is of great appeal for application to stimulate DIET [10, 13].

Foam nickel (FN) functioning as a metallic CM exhibits much higher electrical conductivities ($1.4\times10^{7}$ $S \cdot m^{-1}$) than carbon based CMs and its three-dimensional framework structure facilitates its recovery and reuse. The electrical conductivity of nickel is higher than iron ($1.3\times10^{6}$ $S \cdot m^{-1}$) and its ecotoxicity is much lower than copper ion, [14]. The larger volume fraction of gas-filled pores of FN benefited the development of electroactive biofilm when FN was employed as electrode in microbial fuel cells (MFC) [15]. Given that these superior advantages of FN benefit

for electron transfer, electroactive biofilm development, and feasibility for recovery and reuse, FN may be a promising CM to efficiently stimulate DIET and promote syntrophic methanogenesis. The AD of nonionic surfactants in industrial wastewater is limited to the syntrophic methanogenesis of its important group linear alcohol ethoxylates, where ethanol is the key intermediate and the stimulatory of its DIET can boost the syntrophic methanogenesis [4]. The gap in the state of the art is that the effects of FN supplementation on stimulatory of DIET and microbial community composition shifts benefit for syntrophic methanogenesis have yet to be reported. Moreover, FN is a core of reinforcing material in nickel-hydrogen batteries and is a potential industrial waste after its discarded. As such, this study will provide a novel approach to utilize discarded FN.

In this study, the innovation is for the first time to calibrate the stimulatory of DIET with metal foam (in the case of FN) for methane production improvement. This can provide a novel approach to stimulate syntrophic methanogenesis. The objectives are to evaluate the influence of FN supplementation on biogas production, to investigate the difference in microbial community composition between suspended sludge and biofilm foamed on FN, and to analyze the mechanism of DIET benefit for methane production with FN supplementation.

## 2. Materials and methods

### *2.1. Experimental materials*

The sludge was sourced from a rural household anaerobic digester in Chongqing, stored for degassing for 90 days at room temperature in a plastic container, and subsequently acclimatized on three separate occasions in our laboratory anaerobic digester treating cellulose [16]. The total suspended sludge (TSS) of the inoculum was 77.2 g/L and the ratio of volatile suspended sludge (VSS) to TSS was 53.1%. The FN, characterized by electrical conductivity of $1.4\times10^{7}$ $S\cdot m^{-1}$, 97% porosity and a pore size of 0.3 mm (Kunshan Guangjia New Material Ltd, China) was cut into blocks (10 × 10 × 5 mm, L × W × H) weighting 0.07 ± 0.006 g each.

### *2.2. Batch experiments*

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

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

*2.3. Analytical methods*

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

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 $mV \cdot s^{-1}$ 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

Arch349F (5'-GYGCASCAGKCGMGAAW-3') and Arch806R (5'-GGACTACVSGGGTATCTAAT-3') were used for amplification of the V3-V4 region of archaeal 16S rRNA gene [23]. Illumina HiSeq 2500 was used for 16S rRNA gene sequencing. Raw sequences were assembled, screened, and trimmed. Sequences obtained in the clone libraries were assigned different operational taxonomic units (OTU) and each OTU represented 97% sequence identity. The neighbor-joining phylogenetic trees were constructed to identify the representative OTUs along with searching for the closest matched sequences with the BLAST program in the National Center for Biotechnology Information (NCBI) database. The raw sequencing reads were deposited in the NCBI SRA under project number PRJNA573494 with accession SAMN12811488-SAMN12811490 and SAMN12816961-SAMN12816964.

*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.

## 3. Results and discussion

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

When ethanol was used as a model substrate in AD, the syntrophic methanogenesis of ethanol involves interspecies electron transfer. In this AD system, the hydrogen produced was hard to detect due to the high activities of hydrogenotrophic methanogens. The methane production rate (MPR) and specific methane yield (SMY) in the control are depicted in Figs. 2a and 2b, respectively. As the cumulative duration of the batch experiment increased to 2 days, the MPR rapidly 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 3.5 (Fig. 2b). As the time increased to day 4.5, the MPR quickly increased and peaked at 91.5 mL/g-ethanol/d (Fig. 2a). Given that ethanol was readily degraded into acetate, the accumulation of acetate not only contributed to higher MPR, but also led to a significant drop in pH (Fig. 2c). Methanogens are considered to be sensitive to pH 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. 2a). As further time past (from day 6 to 8 d) the MPR quickly decreased and by day 8 methane production was minimal owing to the depletion of acetate. Maximum SMY of 500.0 mL/g-ethanol was obtained on day 10 (Fig. 2b).

When the FN was supplemented at a concentration of 2.45 g/L (FN 2.45), the trends of SMY and MPR were in line with the control during the initial two days (Figs. 2a and 2b). As the time of experiment increased from day 2 to 3, the MPR quickly

increased to the peak of 94.5 mL/g-ethanol/d, 27.4% higher than the control ($p <$ 0.01). The time to achieve maximum MPR reduced by 33% as compared to that of the control (4.5 d) (Fig. 2a). From day 3 to 4.5, the MPR was almost stable regardless of a lower pH in the reactor, suggesting a high performance with this level of FN supplementation (Fig. 2c). From day 2 to 6 the cumulative methane production was 14.5% higher with FN 2.45 than in the control ($p < 0.01$, Fig. 2a). It is postulated that the enhancement of SMY and MPR may be attributed to the stimulation of DIET induced by FN. Afterwards, the MPR quickly dropped to near zero on day 8 due to the depletion of acetate. Finally, the SMY reached 514.8 mL/g-ethanol on day 10, corresponding to 70.5% of the theoretical value (Fig. 2b). No substantial enhancement on SMY with stimulatory of DIET was observed as compared to the control, which is consistent with previous findings [25]. This may be related to the ready biodegradability of ethanol and the high activities of inoculum.

When the FN concentration was increased from 7.35 to 24.50 g/L, the maximum MPR reduced from 63.7 to 3.7 mL/g-ethanol/d (Fig. 2a), and SMY decreased from 384.2 to 24.0 mL/g-ethanol (Fig. 2b). The inhibition is attributed to the increased dissolution of nickel ion (4.0-4.7 mg/L) in the solution (Fig. 2d). This was consistent with the previous find that overloading of nickel ions suppressed microbial activity and led to the reduction in SMY and MPR [26]. Similar trends of inhibition induced by higher dose of CMs supplementation such as nano-graphene, polyaniline nanorods, and graphene were documented by others [12, 16].

### *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 3. For the first 4 days the ethanol in both the control and FN 2.45 groups was rapidly degraded (Figs. 3a and 3b). At the same time acetate in the control and the FN 2.45 groups increased gradually and the FN 2.45 group peaked at about 37.0 mmol/L on day 4. The accumulation of acetate was consistent with the significant drop in pH (Fig. 3c). Although the trends for ethanol and acetate in the control and FN 2.45 were 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 acetate production with FN 2.45 can be ascribed to the efficient stimulation of DIET 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 consumption of acetate by acetotrophic methanogens for methane production. From day 6 to 10 the acetate in the control and FN 2.45 was depleted, whilst beyond day 7 minimal amounts of methane were produced (Figs. 3a and 3b).

When the FN concentration was increased from 7.35 to 24.50 g/L, acetogenesis was significantly inhibited (Figs. 3c, 3d, and 3e). 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

FN 12.25 and FN 24.50. The degradation of ethanol and acetate was significantly inhibited with FN 12.25 and FN 24.50. This is ascribed to the toxicity of higher concentration of nickel ions to microbes (Fig. 2d).

*3.3 Influence of FN supplementation on microbial community in AD process*

3.3.1 Microbial community in suspended sludge

The original inoculum, suspension from the control, and suspension from optimal FN supplementation group (FN 2.45) termed as FN 2.45 suspension, were collected for investigating microbial community in suspended sludge. Alpha diversity indexes for estimating the microbial richness (i.e. ACE and Chao) and microbial evenness (i.e. Shannon and Simpson) are presented in Table 1. The bacterial richness and evenness in the FN 2.45 suspension were lower than in the control indicating that FN 2.45 supplementation significantly influenced bacterial community. The dominant bacteria in the inoculum and control were *Rikenellaceae* DMER64. In the FN 2.45 suspension *Rikenellaceae* DMER64 and *Dechlorobacter* dominated (Fig. 4a). The relative abundance of *Rikenellaceae* DMER64 in the inoculum, the control, and the FN 2.45 suspension were 23.3%, 11.7%, and 18.7%, respectively, which were higher than in the biofilm adhered to the FN 2.45 carriers (3.2%) (Fig. 4a). *Rikenellaceae* DMER64 is an active member of the bacterial community in common AD systems [29], which may be beneficial for interspecies hydrogen transfer. The relative abundance of *Dechlorobacter* in the FN 2.45 suspension was significantly enriched as compared to the control and the inoculum (Fig. 4b). *Dechlorobacter* 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. 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, *Dechlorobacter*_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 1). The dominant archaea in the inoculum, the control, and the
290 FN 2.45 suspension were *Methanosaeta*, *Methanospirillum*, and *Methanolinea* (Fig.
291 5a). The relative abundance of *Methanosaeta* 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
294 suspension. The abundance of *Methanospirillum* in the control and FN 2.45
295 suspension increased between 4.8 and 9.5 times by comparison to the original
296 inoculum (Fig. 5b). The relative abundance of hydrogenotrophic methanogens,
297 *Methanospirillum* and *Methanolinea*, were influenced with FN supplementation,
298 which is in a similar pattern to previous studies [28, 33]. The enrichment of
299 *Methanospirillum* in the FN 2.45 suspension indicates its potential roles in DIET.
300 Although the archaellum of *Methanospirillum hungatei* was electrically conductive
301 [34], the actual role of *Methanospirillum* in DIET remains elusive as co-cultures of
302 *Methanospirillum* and exoelectrogen via DIET have not been validated.

303 3.3.2 Electrochemical characteristics of biofilm adhered to FN carriers

304 The electrochemical activity of biofilm adhered to the FN 2.45 carriers was
305 assessed by CV analysis (Fig. 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. 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. 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. 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. 2a).

320

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

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

due to the preferences of FN 2.45 to exoelectrogens (Table 1). The dominant bacterial genera in the FN 2.45 biofilm were *Clostridium sensu stricto*, *Acinetobacter*, and *Dechlorobacter* (Fig. 4a). The *Clostridium sensu stricto* in the FN 2.45 biofilm accounted for 29.6% relative abundance, which is significantly higher than in the control and FN 2.45 suspension (Figs. 4a and 4b). The *Clostridium sensu stricto* was also significantly enriched when conductive polyaniline nanorods was added to stimulate DIET [12]. *Clostridium*, a genus of gram-positive bacteria, was shown to be electrochemically active [37, 38]. The enriched *Clostridium sensu stricto* accordingly may facilitate DIET with methanogens in the FN 2.45 biofilm. *Clostridium sensu stricto* 11 accounting for 19.7% relative abundance was enriched significantly as compared to that in the inoculum, the control and the FN 2.45 suspension (Fig. 4b). *Clostridium sensu stricto* 11_OTU3 was closely affiliated to *Clostridium guangxiense* strain xsk1 and *Clostridium guangxiense* strain ZGM211 (100% sequence identity) (Fig. 4c). *Clostridium guangxiense* ZGM211 is a hydrogen-producing strain [39, 40] and might shift its metabolic process from producing $H_2$ to $H^+$ and electrons when conductive FN 2.45 is present. The remarkable enrichment on *Clostridium sensu stricto* 11_OTU3 in FN 2.45 biofilm implied it played a vital role in DIET. *Acinetobacter*_OTU8, *Acinetobacter*_OTU373 and *Acinetobacter*_OTU653 were related to *Acinetobacter* sp. strain 18H6A8 (Fig. 4c). *Acinetobacter* was reported as an electroactive genus in Mn oxidizing/reducing and is closely related to the extracellular electron transfer process [41, 42]. Therefore, the significant enrichment of *Acinetobacter* in the FN 2.45 biofilm (i.e. 4.2 times higher compared to the control)

revealed its potential contribution to DIET in the AD process.

The archaeal evenness in the FN 2.45 biofilm was lower than in the control probably due to more electrotrophic methanogens in the biofilm than in the suspension. The dominant archaea in the FN 2.45 biofilm were *Methanosaeta*, *Methanospirillum*, and *Methanolinea* (Fig. 5a). The abundance of *Methanosaeta* in the FN 2.45 biofilm increased by 75.8% as compared to the original inoculum. Compared to the control, the relative abundance of *Methanosaeta* in the FN 2.45 biofilm was enriched by 14.1%. *Methanosaeta harundinacea* (JCM-13211) was reported to enable DIET with *Geobacter metallireducens* [6]. The same pattern that *Methanosaeta* was enriched with CMs supplementation to stimulate DIET was also reported [43, 44]. The enhancement of *Methanosaeta* in response to CMs supplementation revealed the stimulatory of DIET in the AD process. Nevertheless, decrease in *Methanosaeta* with CMs supplementation has also been documented [16]. The variation in these findings is ascribed to the difference of feedstocks and rector configuration. *Methanosaeta*_OTU1 was closely affiliated to *Methanosaeta concilii* GP-6 (100% sequence identity) and *Methanosaeta harundinacea* (JCM-13211) (Fig. 5c). *Methanosaeta*_OTU64 was affiliated to *Methanonethylovorans hollandica* DSM 15978 (98.4% sequence identity) and *Methanosarcina barkeria* DSM800 (Fig. 5c). Hence, the enhancement on *Methanosaeta* in the FN 2.45 biofilm as compared to the control implied that *Methanosaeta* enable to conduct DIET with exoelcetrogens.

*3.4 Integrated interspecies electron transfer with FN supplementation*

The syntrophic consortia involved in interspecies electron transfer with FN 2.45 supplementation is shown in Fig. 7. The enrichment on syntrophic consortia such as *Geobacter*, *Methanosarcina*, and *Methanosaeta* with CMs supplementation indicated the stimulatory of DIET [45, 46]. However, the syntrophic consortia capable of direct interspecies electron transfer are not limited to the aforementioned genera.

Based on the microbial community shifts in the control, the FN 2.45 biofilm, and the FN 2.45 suspension (Fig. 7) as well as the functions reported in previous works, the potential syntrophic consortia capable of DIET and MIET are discussed. The potential hydrogen-producing bacteria for MIET was *Rikenellaceae* DMER 64 due to its relative abundance significantly higher than in the FN 2.45 biofilm. *Rikenellaceae* DMER 64 transferred the electron carrier $H_2$ to hydrogenotrophic methanogens (i.e. *Methanospirillum* and *Methanolinea*). Both the relative abundance of *Methanospirillum* in the FN 2.45 suspension and *Methanolinea* in the control were higher than those in the FN 2.45 biofilm, suggesting that MIET played an important role in the suspension. The potential exoelectrogens in DIET were *Acinetobacter*, *Clostridium sensu stricto* 11 and *Dechlorobacter* due to their enrichment with FN 2.45 supplementation. *Acinetobacter* and *Clostridium sensu stricto* 11 were the predominant exoelectrogens in the FN 2.45 biofilm. *Dechlorobacter* were the predominant exoelectrogens in the FN 2.45 suspension. These potential exoelectrogens produced electrons in acetogenesis and transferred it to *Methanosaeta*, which reduced $CO_2$ with electrons to produce methane and metabolized acetate to

methane as well. The relative abundance of exoelectrogens in the FN 2.45 biofilm was higher than that in the control, indicating DIET played an important role in the FN 2.45 biofilm and MIET was dominant in the control. The higher abundance of *Methanosaeta* also indicated DIET dominance in the FN 2.45 biofilm.

DIET and MIET generally coexist in the AD process, whereas their contribution ratio to interspecies electron transfer is influenced by not only the characteristics of CMs, but also the fermentation conditions including microbial activity, substrate biodegradability, and rector configuration [47, 48]. Previous studies suggested DIET predominated in up-flow anaerobic sludge blanket reactors due to the sludge bed facilitating syntrophic consortia to form biofilm [6]. Moreover, interspecies electron transfer in the AD system with conductive carbon cloth supplementation was postulated to shift from interspecies hydrogen transfer to DIET [49]. The relative abundance of *Geobacter* attached on conductive carbon cloth was more than that in suspended sludge [49]. Therefore, it is postulated that biofilm is more favorable for syntrophic consortia to perform DIET than suspended sludge. This is explained in two ways: 1) biofilm keeps consortia tightly in contact and decreases the distance for electron transfer; 2) the conductive matrix of biofilm facilitates interspecies electron transfer [50]. This was in accordance with the evidence that more exoelectrogens and electrotrophic methanogens were present in the FN 2.45 biofilm as compared to that in the control. Therefore, biofilm adhered to CMs is also a favorable source to investigate the syntrophic consortia. Apart from FN 2.45 facilitating formation of 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 utilization and methane production via reducing energy consumption for synthesis of OmcS and pili.

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

### *3.5 Trends and Perspectives*

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

[52]. The Ministry of Ecology and Environment of the People's Republic of China set the national wastewater discharge standard for 12 different industrial sectors [53]. AD can efficiently degrade the organic matter in industrial wastewater for biogas generation and wastewater treatment. In 2019, the National Development and Reform Commission set a target of annual production of bio-natural gas exceeding 10 billion 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 efficiently boost AD performance in terms of an increase in specific methane yield by 11.2% on day 6 and the maximum methane production rate by 27.4. Allowing a 10% enhancement in methane yield during AD of dye wastewater with an HRT of 6 d at FN concentration of 2.45 kg/$m^3$, only 1.4 years is expected to recover the investment of supplemented FN due to the extra methane production.

To demonstrate the enhancement effect of FN during the AD of real industrial wastewater in full-scale, a long-term continuous experiment using dye wastewater or papermaking wastewater in an upflow anaerobic sludge blanket reactor is necessary to fully assess the impacts of FN supplementation on methane production. Moreover, the modification of the surface properties of FN such as an increase in biocompatibility or roughness to enhance biofilm formation may be beneficial for further stimulatory of DIET.

## 4. Conclusion

When FN was supplemented at a concentration of 2.45 g/L, a 27.4% increase in maximum MPR was observed on day 3; the time to maximum MPR reduced by 33% as compared to the no FN group. The electroactive biofilm formed on FN 2.45 carriers (FN 2.45 biofilm) could generate electrons. The potential exoelectrogens *Clostridium sensu stricto* and dominant methanogen *Methanosaeta* were enriched significantly benefit for DIET in the FN 2.45 biofilm. FN supplementation would have a significant potential to stimulate DIET and accelerate the syntrophic methanogenesis during the AD of industrial wastewater.


## Acknowledgements

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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 transfer | OmcS | outer membrane c-type cytochrome |
| FN | foam nickel | OTU | operational taxonomic units |
| FN 2.45 | the group with FN added at the concentration of 2.45 g/L | PBS | phosphate-buffered saline |
| FN 2.45 biofilm | biofilm adhered to FN 2.45 carriers | SEM | scanning electron microscopy |
| FN 2.45 suspension | suspension from optimal FN supplementation group | SMY | specific methane yield |
| GC | gas chromatography system | TSS | total suspended sludge |
| ICP-OES | inductively coupled plasma-optical emission spectroscopy | VFAs | volatile fatty acids |
| MIET | mediated interspecies electron transfer | VSS | volatile suspended sludge |

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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.

**Fig. 5** (a) Archaeal 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.

**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 2.45 carriers and FN control, respectively.

**Fig. 7** Syntrophic consortia involved in interspecies electron transfer.

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

| | 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 |

[a] Foam nickel

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

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

661 [a] conductive material; [b] Methane production rate; [c] Reactive Red 2; [d] Not available; [e] Waste
662 activated sludge; [f] Zero-valent iron; + significant increase.
663

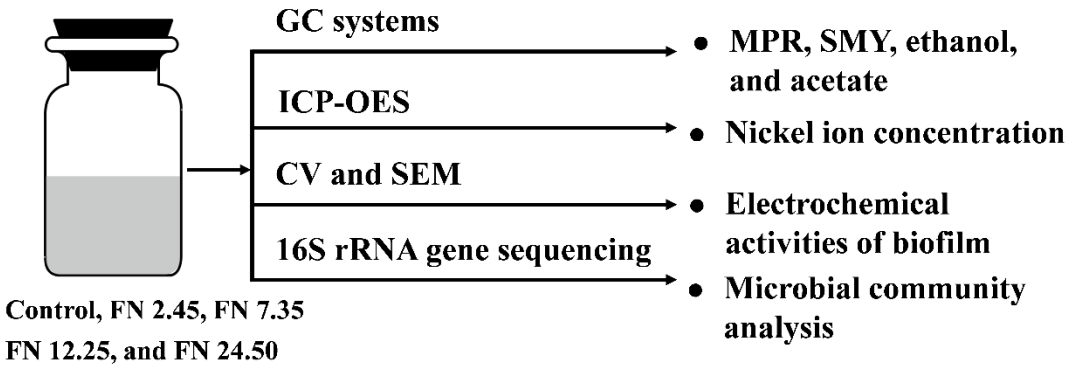



**Fig. 1** The experimental scheme of stimulatory of DIET with FN supplementation. MPR and SMY denote methane production rate and specific methane yield, respectively.

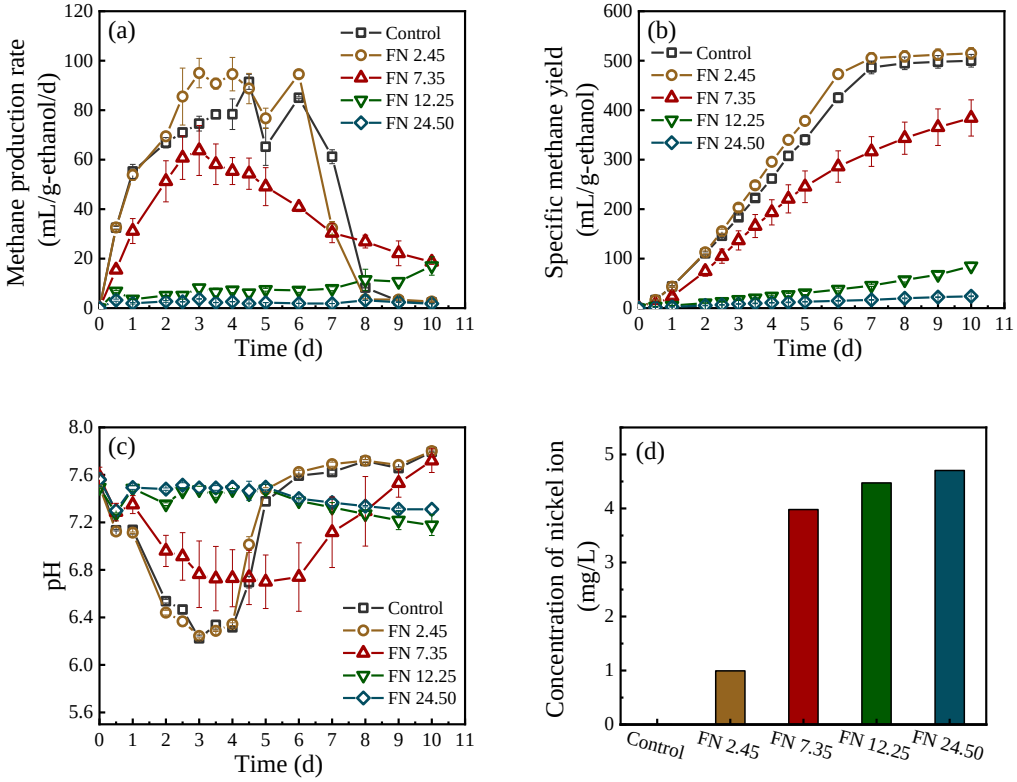
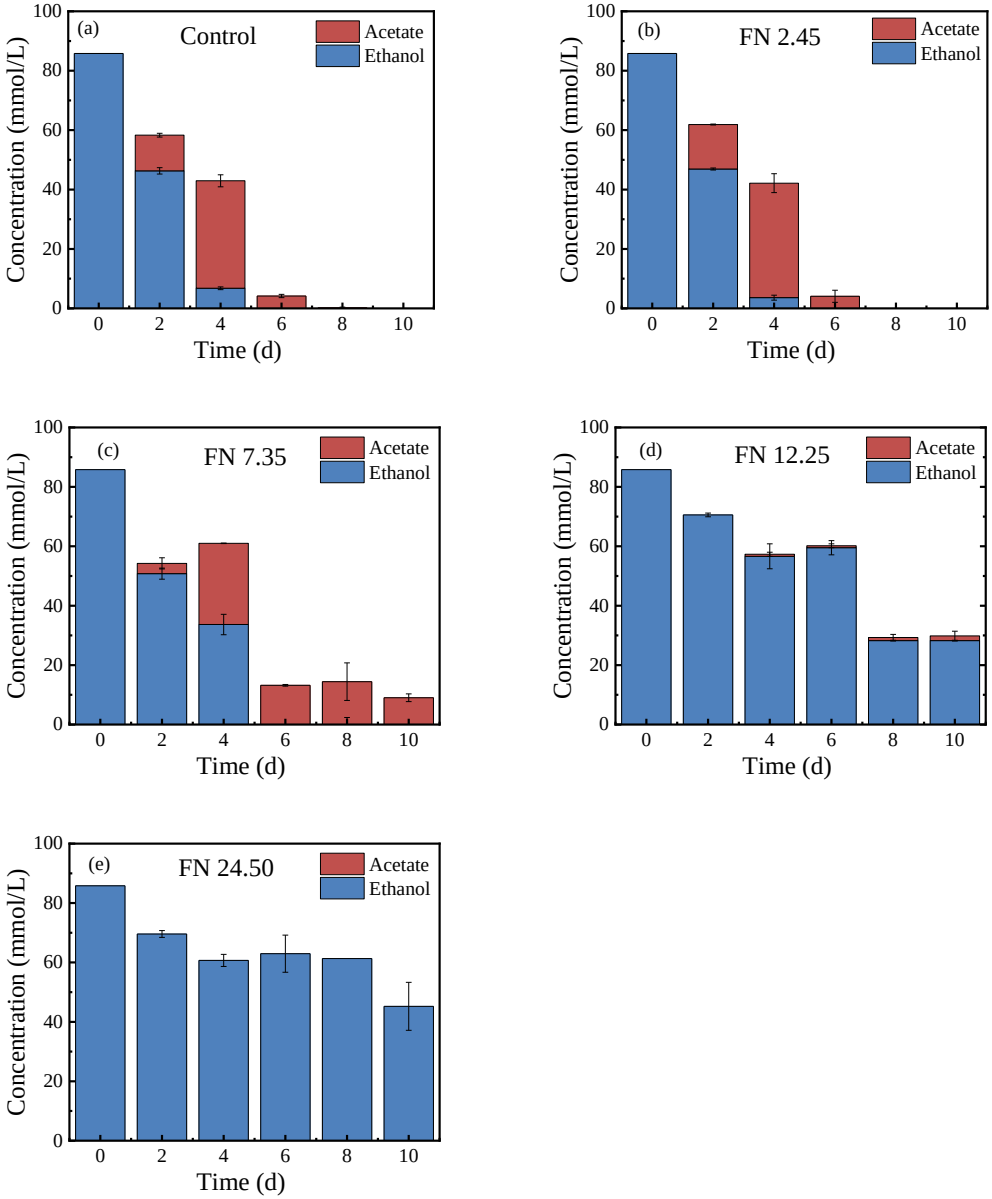



**Fig. 2** Influence of FN supplementation on (a) methane production rate, (b) specific methane yield, (c) pH changes, (d) concentration of nickel ion after AD. Each value is an average of triplicate assays, and each bar indicates ± standard deviation (n=3).




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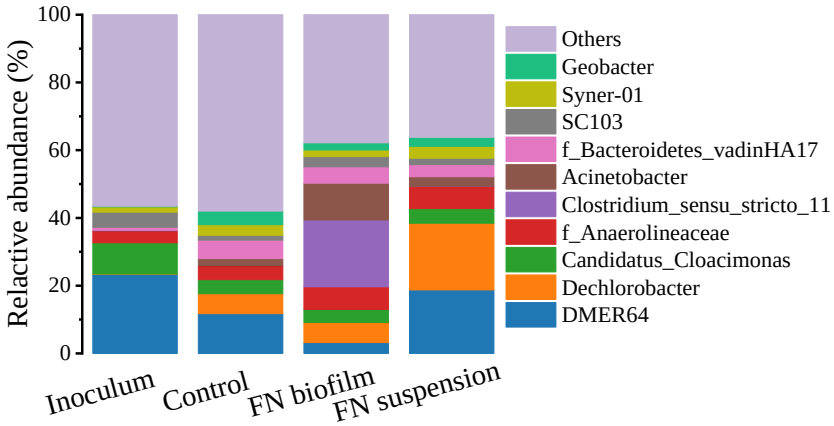
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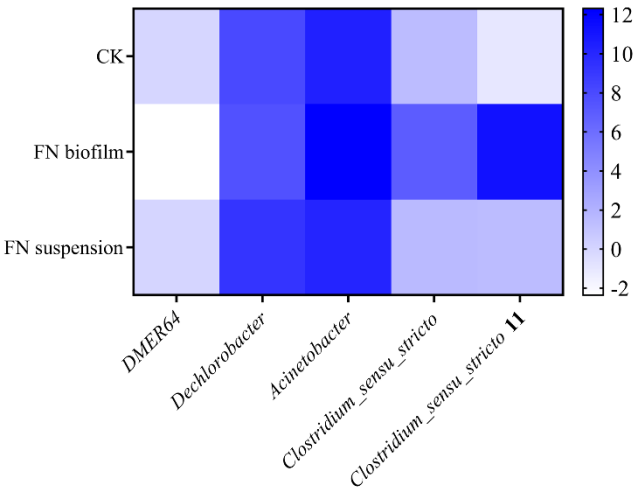
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681 **Fig. 3** Influence of FN supplementation on the concentrations of ethanol and acetate
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683 24.50. Each value is an average of triplicate assays, and each bar indicates ± standard
684 deviation (n=3).

(a)




(b)




(c)

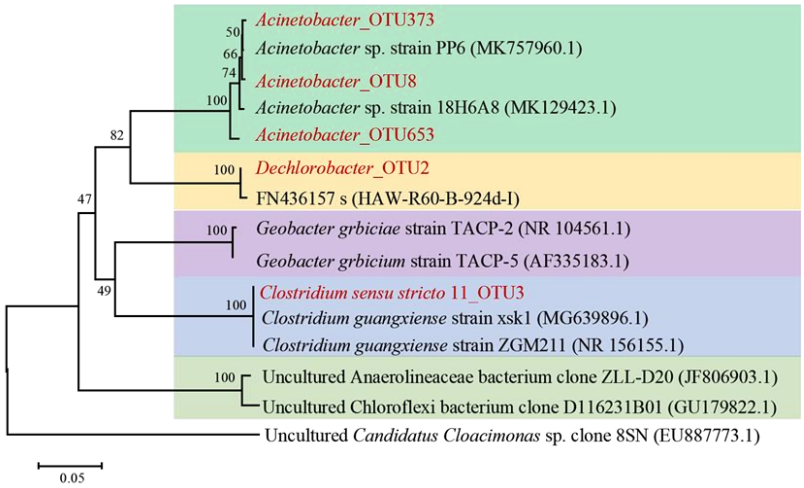
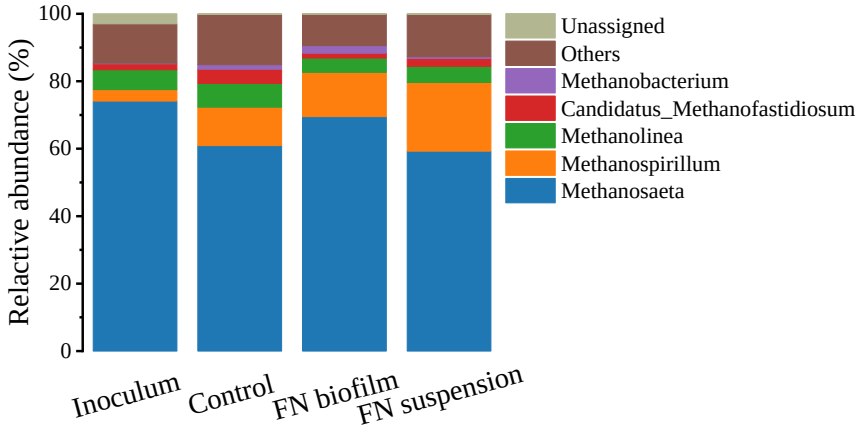
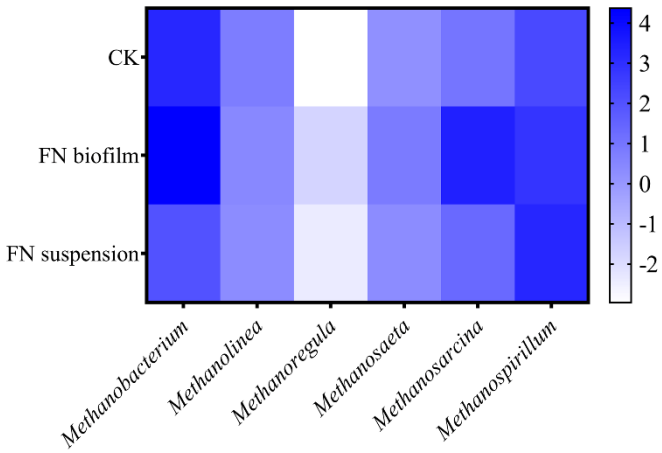



**Fig. 4** (a) Bacterial community structure at genus level after AD, (b) $Log_2$ fold change of abundance of dominant genera in the control, FN 2.45 biofilm, and FN 2.45 suspension to inoculum, (c) Neighbor-joining phylogenetic tree of representative species.

(a)




(b)




(c)

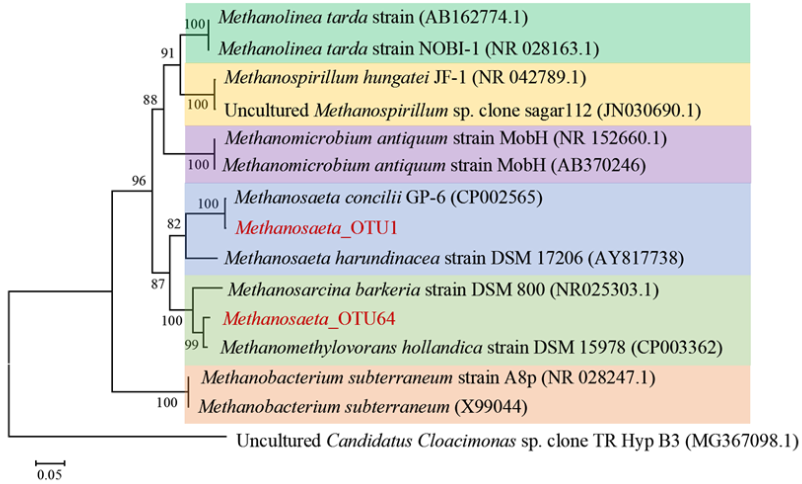
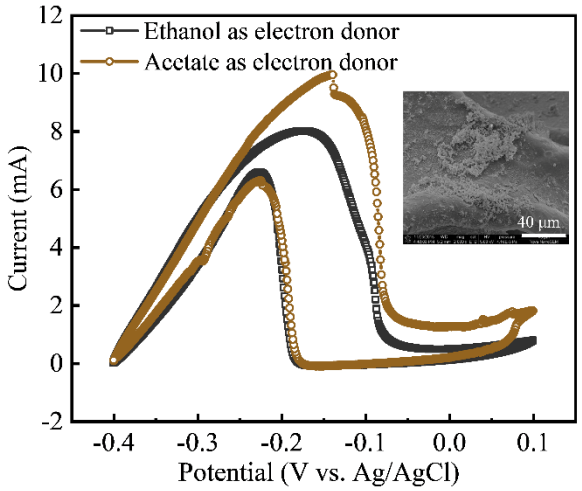



**Fig.** 5 (a) Archaeal community structure at genus level after AD, (b) $Log_2$ fold change of abundance of dominant genera in the control, FN 2.45 biofilm, and FN 2.45 suspension to inoculum, (c) Neighbor-joining phylogenetic tree of representative species.

(a)




(b)

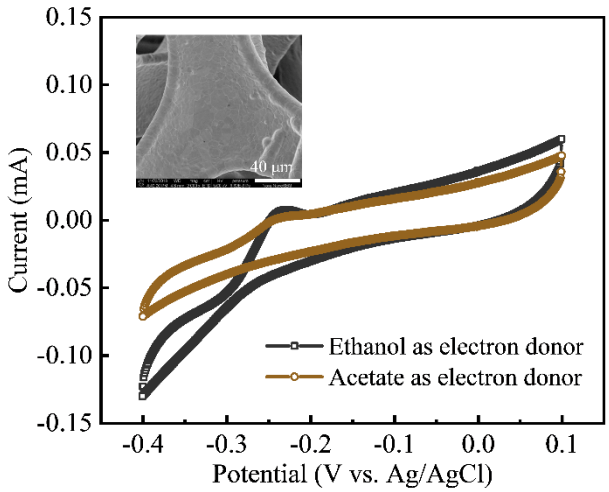
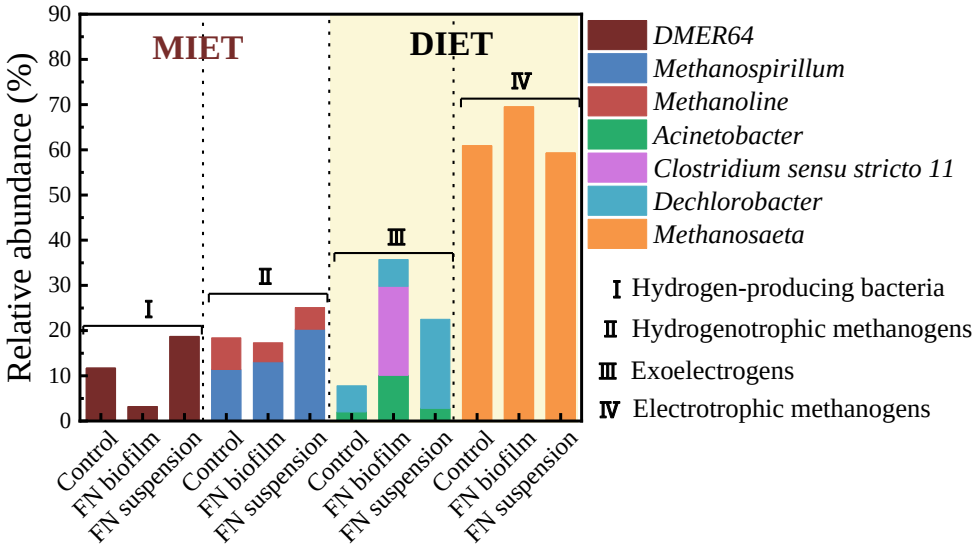



**Fig. 6** Cyclic voltammogram measured in the single chamber MFC. (a) FN 2.45 carriers as anode, (b) FN control as anode. Insets for a and b show SEM images of FN 2.45 carriers and FN control, respectively.




714

715 **Fig. 7** Syntrophic consortia involved in interspecies electron transfer.

***Abstract**

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.

***Credit Author Statement**

**Xiaobo Guo and Chihe Sun:** Data curation, Writing- Original draft preparation. **Ao Xia:** Conceptualization, Supervision. **Richen Lin:** Investigation. **Yun Huang and Xianqing Zhu:** Visualization and Software, **Pau-Loke Show and Jerry D. Murphy:** Writing- Reviewing and Editing.