Enhanced anaerobic phenol degradation by conductive materials via EPS and microbial community alteration

Yan, Wangwang; Sun, Faqian; Liu, Jianbo; Zhou, Yan

2018

Yan, W., Sun, F., Liu, J., & Zhou, Y. (2018). Enhanced anaerobic phenol degradation by
conductive materials via EPS and microbial community alteration. *Chemical Engineering
Journal*, 352, 1-9. doi:10.1016/j.cej.2018.06.187

<https://hdl.handle.net/10356/136937>

<https://doi.org/10.1016/j.cej.2018.06.187>

© 2018 Elsevier B.V. All rights reserved. This paper was published in *Chemical Engineering
Journal* and is made available with permission of Elsevier B.V.

Downloaded on 26 Apr 2023 16:31:40 SGT

Enhanced anaerobic phenol degradation by conductive materials via EPS and microbial community alteration

Wangwang Yan ^{a, b}, Faqian Sun ^b, Jianbo Liu^b, Yan Zhou ^{a, b *}

^a *School of Civil and Environmental Engineering, Nanyang Technological University, 639798, Singapore*

^b *Advanced Environmental Biotechnology Centre, Nanyang Environment and Water Research Institute, Nanyang Technological University, 637141, Singapore*

*Corresponding Author: Yan Zhou

Address: 50 Nanyang Avenue, Singapore 639798

E-mail: Zhouyan@ntu.edu.sg

Tel: (+65) 67906103

Fax: (+65) 67910676

Abstract

Anaerobic biodegradation is a promising and economical process to remove phenol in the wastewater, although the reaction kinetics are often limited by the toxicity of phenol. This study proposed a strategy to accelerate phenol degradation and mitigate its inhibition on the bioprocess by adding conductive materials (CMs) into the anaerobic degradation system. The results showed that CMs could stimulate phenol degradation up to one-fold higher than that in control group. Certain extracellular polymeric substances (EPS) compounds that can act as electron shuttles, i.e. protein and humic substances, were greatly enriched with the existence of CMs. In particular, compared with control group, CMs supplemented groups had 2.3 and 10 to 20 folds low molecular weight protein in bound and soluble EPS, respectively. Carbon nanotube supplemented group exhibited 3 times higher humic substance in soluble EPS. The overall electron transport system activity in CMs groups was much higher than that in control groups. The addition of CMs enriched phenol degradation bacteria—*Syntrophorhabdus*, *Brooklawnia*, *Treponema* and *Syntrophus*, as well as electroactive methanogens—*Methanosaeta*. It is proposed that *Syntrophus* and *Methanosaeta* were the functional genera in methanogenic phenol degradation via extracellular electron transfer. This study revealed that the presence of CMs altered the EPS composition and microbial community of the system and made it become more favorable for extracellular electron transfer.

Keywords: Phenol degradation; Extracellular polymeric substances (EPS); Electron shuttles; Humic substance (HS); Extracellular electron transfer (EET); Microbial community

1. Introduction

Phenol is a common toxic compound in industry effluents. If it is discharged to open water bodies directly, serious ecological problems would be induced. Compared to physical, chemical and aerobic biodegradation treatment methods, anaerobic biological phenol degradation shows great advantages in terms of cost and efficiency [1]. The complete anaerobic phenol degradation can be carried out through cooperation between phenol-degrading bacteria and methanogens [2], and the syntrophic methanogenesis is performed via adopting molecular intermediates, typically hydrogen [3]. Recent studies show that direct extracellular electron transfer (EET) widely exists among syntrophic partners [4], and can be triggered by addition of conductive materials (CMs) [5, 6]. In addition, theoretical analysis reveals that electron transfer via CMs could be around 10^6 times higher than that associated with interspecies hydrogen transfer, and certain organic compounds degradation could be stimulated via EET [7-10]. These facts strongly suggest the feasibility of CMs to stimulate anaerobic phenol degradation by triggering direct EET between syntrophic partners.

The enrichment of electroactive microorganism is required for conducting EET. Currently, CMs, typically carbon-based and iron-based materials, are widely reported with the capability of altering anaerobic microbial community for promoting EET [9, 11]. Nevertheless, till now, limited studies compared the effect of different CMs on anaerobic microbes. In this study, single-walled carbon nanotube and magnetite nanoparticles are chosen as representative CMs to compare their effect on phenol degradation efficiency and microbial community structure.

Extracellular polymeric substances (EPS) are ubiquitous heterogeneous mixtures of natural

organic macromolecules. It can form a protective layer for microbial cells [12], with the thickness up to 1 μm [13, 14]. Humic substance and redox protein within EPS matrix are widely reported to be electrochemically active [12, 15]. Large number of functional groups in EPS, such as ketone, carboxylic acid, phenolic and alcoholic hydroxyls, can absorb/bind secreted redox protein such as flavoprotein to promote EET [12]. CMs promoted syntrophic electron transfer are based on applying CMs as conduit, where direct contact between cells and CMs are needed [7, 16]. Since CMs likely interact with EPS layer first to establish contact with cells, it is important to explore the role of EPS in EET, which, however, has been ignored by most studies. To date, it is not clear if the EPS compositions would be influenced by the presence of CMs, and if EPS acts as competitor or compensator of CMs to facilitate EET. In this study, the influence of CMs on EPS distribution, and the interaction between them as well as the role of EPS in EET were examined and discussed.

2. Materials and methods

2.1 Experimental setup and operation

The seed sludge for anaerobic batch reactors was collected from a lab-scale mesophilic (35°C) continuous stirred-tank reactor (CSTR) fed with glucose (107 mg/L COD), phenol (238 mg/L COD), and trace element. The composition of the trace element was as follows: NH_4HCO_3 , 410 mg/L; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 210 mg/L; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg/L; $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 4.5 mg/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mg/L; NaCl, 10 mg/L; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 5 mg/L; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 39 mg/L; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 5 mg/L; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5 mg/L; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mg/L; ZnCl_2 , 5 mg/L; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mg/L; H_3BO_3 , 8 mg/L.

All batch experiments were conducted using AMPTS II system (Bioprocess Control

Company) to track the methane production automatically. Three batch experiment groups, namely, control group (C), magnetite nanoparticles (M) supplemented group and single-walled carbon nanotube (CNT) supplemented group, were setup in triplicates each. Each batch reactor had a working volume of 360 mL with a final volatile suspended solids (VSS) concentration of 3.11 g/L. CNT (0.30 g CNT/g VSS) or magnetite particles (1.75 g magnetite/g VSS) was added into corresponding supplemented batch reactors. Reactors were purged with nitrogen gas for 10 mins to remove dissolved gas. All batch reactors were operated in semi-continuous mixing mode with 90 s on and 90 s off, and stirring speed was 80 rpm.

The phenol degradation experiment lasted for 165 days that can be divided into six phases. Detailed operation condition and sampling time can be found in Table 1. In the last feeding cycle of phase 3 (day 72), before feeding, all reactors were exposed to air for 60 mins to examine the influence of oxygen on the process. Phenol degradation performance were closely monitored at the first, middle and final feeding cycles in each FPC phase. The biomass for microbial community analysis was collected at the end of each FPC phase. Homogeneous sludge samples were taken out on day 160 for analyzing soluble iron ions (II and III) from all reactors.

The pretreatment method of single-walled CNT and its physicochemical property can be found in Yan et al. [17]. Magnetite nanoparticles were synthesized according to Kang et al. [18]. X-ray diffraction (XRD) and transmission electron microscope (TEM) were employed to confirm the product as magnetite nanoparticles (Fig. S1 and S2).

2.2 Analytical methods

VSS was measured according to Standard Methods [19]. VFAs were measured by a gas chromatograph (GC) according to a previously described method [17]. Phenol concentration was

measured by a high-performance liquid chromatography (HPLC) system (Shimadzu LC-20AD, Japan). The HPLC was equipped with an Agilent Proshell 120 EC-C 18 column (4.1×100 mm, 2.7 µm) and an SPD-M20A diode array detector. The sample preparation and analysis for iron (II), iron (III), and total iron followed Standard Methods [20].

2.2.1 Electron transport system activities analysis

Electron transport system (ETS) activity of the anaerobic sludge was measured by using INT (2-(p-iodophenyl)-3-(pnitrophenyl)-5-phenyltetrazolium chloride) method [21]. This method is based on analysis of the dehydrogenase activity of anaerobic bacteria, which is correlated with respiratory activity. Briefly, 1 mL homogenized sludge sample, 2 mL Tris-HCl (pH 8.4) and 1.5 mL INT (0.2%) were added into a 10 mL centrifuge tube. After incubating at 37°C for 45 min in dark, 1 mL formaldehyde (37%) was added to terminate the reaction. Then, 5 mL acetone was mixed thoroughly into the tube. The mixture was centrifuged at 5000 g for 5 min, and the absorbance of supernatant was determined at 485 nm.

The INT-ETS activity was calculated using Equation 1:

$$U = \frac{D_{485} V e}{K V T} \quad \text{Eq. 1}$$

Where U is the ETS activity (ug/(mg·min)); D_{485} is the absorbance of the supernatant at 485 nm; V_e is the volume of the solvent; K is the slope of the standard curve; V is the volume of the sludge sample; T is the incubation time.

2.2.2 Methane production analysis

The OriginPro software (version 8.5) was used to fit cumulative CH₄ data (collected by AMPTS system) to a modified Gompertz three-parameter model (Eq. 2) [22].

$$M(t) = P \times e \left\{ -e \left[\frac{R_{max} \times e}{P} \times (\lambda - t) + 1 \right] \right\} \quad \text{Eq. 2}$$

Where $M(t)$ is cumulative methane production (mL) at time t (h); P is the potential maximum CH_4 yield (mL), calculated based on theoretical value of 350 mL CH_4 / g COD; R_{max} is the maximum CH_4 production rate (mL/h); λ is the lag phase (h).

Maximum methane production rate (R_{max}) of first, middle and final reaction cycle under each phase was calculated by using above modified Gompertz three-parameter model.

2.2.3 DNA extraction and microbial community analysis

An automated DNA extraction kit (MP Biomedicals, Singapore) was used for DNA extraction. Primer 515F (5'- GTGCCAGCMGCCGCGGTAA-3') and 806R (5'- GGACTACNNGGGTATCTAAT-3') were used for targeting V3-V4 regions of both bacteria and archaea 16S rDNA genes. After removing the low-quality sequences, adapter primers, barcodes and primers, the result sequencing was performed on an Illumina MiSeq platform by MacroGen Inc. (Korea). Operational taxonomic units (OTUs) were defined by clustering at a 0.03 distance level (97% similarity). All OTUs were analyzed using Ribosomal Database Project to cluster them into related species.

The Chao1 richness estimator, Shannon and Simpson diversity indices, and Good's coverage were calculated in the Mothur software program for each sample based on the OTUs. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree based on bacterial and archaeal 16S rRNA gene sequences was constructed to interpret the microbial structure difference among all samples by using the Quantity One software.

2.2.4 EPS extraction and analysis

A modified heat extraction method was applied to extract soluble, loosely bound, and tightly bound EPS [23]. A liquid chromatography-organic carbon-organic nitrogen detection (LC-OCD-OND) system, equipped with three online detectors, namely organic carbon detector,

UV detector, and organic nitrogen detector, was used to quantify different compounds by continuous analyze the relative signal response of organic carbon, UV and organic nitrogen. The identified organic fractions were mainly divided into two parts, i.e. hydrophobic and hydrophilic dissolved organic carbon (DOC). The hydrophilic DOC was subdivided into biopolymers, high molecular weight protein-like substances (including high molecular weight (HMW) protein, and carbohydrate), HS, building blocks, low molecular weight (LMW) neutrals, and LMW acids. The concentration of these fractions was quantified by a software (ChromCALC, DOC-LABOR, Karlsruhe, Germany), and the unit was mg equivalent carbon per liter (mg-C/L).

Total polymeric substance was measured with phenol-sulphuric acid method [24]. Total protein was determined with the modified Lowry method [25]. The concentration of LMW protein/carbohydrate was calculated by subtracting HMW protein/carbohydrate from total protein/carbohydrate. To unify the units, 1 g of polysaccharide or protein equals 0.444 and 0.497 g of equivalent carbon, based on chemical formulae of polysaccharide $(C_6H_{10}O_5)_n$ and protein $C_{52.5}H_{6.65}N_{16}O_{21.5}S_2$.

3. Results and discussion

3.1 Phenol degradation and methane production performance at different FPC

Fig. 1 exhibits the phenol degradation profile of final feeding cycle in each phase, and the corresponding methane production profile is shown in Fig. 2. Based on the performance, the influence of FPC and CMs on methanogenic activity could be categorized into three stages. The first stage took place in phase 1, when co-substrate was used. Phenol was completely degraded in 60 hours (Fig. 1a). The degradation rates in all groups were similar (2.17 ± 0.01 mg/h). No significant difference was found for R_{max} in all groups (2.11 ± 0.10 mL/h, Fig. 3 P1).

The second stage was from phase 2 to 3 (FPC from 200 – 400 mg/L). In general, phenol degradation and methane production rates increased with the acclimation time and FPC. In phase 2, phenol degradation rates in all groups increased to 10.57 ± 0.53 mg/h (Fig. 1b). It is noteworthy that R_{max} , on the other hand, exhibited a great difference among CMs and control groups, e.g. CNT groups (6.17 mL/h) vs. control groups (4.11 mL/h) (Figs. 2b and 3). From phase 3 onwards, the superiority of CMs enlarged with the increased FPC. In particular, in the middle feeding cycle of phase 3, 2-fold R_{max} was observed in CMs group compared to control groups (8.36 mL/h in CNT and 7.06 mL/h in magnetite vs. 4.29 mL/h in control) (Fig. 3). Besides the improved R_{max} , faster phenol degradation rate was also observed in CMs groups (Fig. 1c).

The third stage was from phase 4 to 6 (FPC 600 mg/L to 1000 mg/L). During this stage, both phenol degradation rate and R_{max} declined with the increase of FPC, and all the reactors could not reach the best performance as before (Figs. 1, 3 and Table S1). Phenol is an inhibitory substrate. Here, the unrecovered activity could be attributed to the inhibition. This result was consistent with previous studies, which reported anaerobic digestion can be inhibited at 400 to 600 mg/L phenol [1, 26]. Nevertheless, CMs groups still performed better than control groups. It is noteworthy that a lag phase was observed in control groups when FPC was 600 mg/L or higher, while a linear regression of phenol concentration was always observed in the CMs groups (Figs. 1d, 1e, and 1f). Together with the methane production profile (Fig. 2), the results indicated that high phenol concentration may still have inhibitory effects in control groups, whereas the CMs groups reached a suboptimal condition after one-month acclimation under each FPC condition.

It is noteworthy phenol degradation rate was much slower in phase 1 than in phase 2. According to Movahedin et al. [27], the influence of co-substrate on phenol degradation varied

with acclimation methods and microbial community. Microorganism may choose to degrade glucose first, and then use phenol as alternative substrate, thus co-substrate may limit phenol degradation. The improvement of R_{max} in CMs groups started from phase 2 (Fig.2 and 3), whereas phenol degradation in CMs groups was only accelerated from phase 3 (Fig. 1). It seemed phenol degradation and methane generation processes decoupled and the activity of methanogens were enhanced earlier. CMs, as electron shuttle to facilitate electron transfer among syntrophic partners, theoretically would not promote the metabolic activity of microbial electron donors directly. At low FPC (200 mg/L), the facilitated phenomenon was only observed in methanogenesis rather than in phenol degradation process. However, at FPC above 400 mg/L, the facilitated methanogenesis could speed up the consumption of upstream accumulated metabolites, thus resulting in enhancement of phenol degradation.

In the last feeding cycle of phase 3, all reactors were exposed to air for 60 mins to investigate the robustness of the systems with and without the presence of CMs. It has been reported that molecular oxygen once entered anaerobe cells can accept one electron from microbial electron respiratory chain and reduced to oxygen free radicals. As anaerobes are lack of superoxide dismutase, free radicals cannot be removed timely, which leads to inhibition on anaerobic metabolism and enzymatic systems [28]. The results showed the methane production rate declined by 46% in control groups, whereas the observed decline in magnetite and CNT supplemented groups were only 17% and 29%, respectively (Fig. 3). The existence of CNT can create a more negative redox potential in the anaerobic microcosms [29], hence, such reduced environment may efficiently mitigate the impact from oxygen. As for magnetite supplemented group, the presence of high concentration of Fe(II) ions can act as electron donor to remove dissolved oxygen [30]. Therefore, the dosed CMs to some extent could resist oxygen triggered

oxidative stress, and maintain a less affected environment for anaerobes.

3.2 Electron transport system (ETS) activities

ETS activity reflects the dehydrogenase activity of the microbial communities, which is associated with biological respiratory activity [8]. The ETS activity in CMs groups was much higher than that in control group during 120 h anaerobic reaction (Fig. 4). In the first hour after feed, the ETS activity of CMs groups was about 10 times higher than that in control group (average $28.05 \mu\text{g}/(\text{min} \cdot \text{mL})$ in CMs VS $3.67 \mu\text{g}/(\text{min} \cdot \text{mL})$). Although the difference narrowed at the end of the cycle when the substrate was fully consumed, the CMs groups still showed about 2 times higher ETS. ETS activity results proved the facilitated electron transfer system with CMs addition.

3.3 EPS alternation and its role in EET

Thermodynamic analysis revealed the feasibility of direct EET via applying CMs as conduit, where direct contact between cells and CMs is needed to carry out this process [31]. TEM observation showed that microbes tend to contact with CNT, or their surface would be covered by dense magnetite nanoparticles (Fig. S2). In addition, the enrichment of electroactive genera was observed in CMs groups, which will be discussed in the later part. These facts strongly indicate the successful establishment of EET condition.

As EPS acts like a capsule of cells, CMs probably would be in contact with EPS of the cells firstly. The electronactive moieties in EPS could play an important role in the EET process [32, 33]. In this study, the presence of CMs clearly influenced all EPS compositions, especially protein and HS (Table 2), which were closely related with the EET capability.

Protein profile was substantially different in CMs and control groups. In particular, around 2.3-fold protein content was observed in bound EPS of CMs groups. CMs could facilitate the expression of redox active protein, such as cytochrome c oxidase which is an essential component of the electron transport chain [34]. Ren et al. [35] also reported that CNT could enhance the enzyme activity of horseradish peroxidase by binding to its activity center, with clear observation of electron transfer using near-infrared spectroscopy. Both magnetite and CNT would rely on direct contact with protein active center for accepting or donating electron [32]. Given spatial distribution of those proteins, the higher quantity of protein that bound to cells may assist in better electron transfer between cells and CMs. Therefore, the facilitated EET between syntrophic microbes was likely fulfilled by applying CMs as mediator to accept and donate electrons from electro-active protein.

On the other hand, LMW protein from soluble EPS was 10 to 20 folds higher in CMs (105.77 - 234.83 mg-C/L) groups than control group (12.27 mg-C/L) (Table 2). It has been reported that some redox proteins (e.g. flavoprotein) involved in electron transfer during cell metabolism processes could be released into extracellular environment, and remain high electrochemical activity that can promote long distance electron transfer [12, 36]. It is reasonable to predict that greater amount of protein within soluble EPS in CMs may also play a role in carrying out long distance interspecies electron transfer.

Building blocks are the break-down products or small fractions of humic substances. Building blocks and humic-like substance are combined called humic substance (HS) hereafter. The distribution of humic substance was very different in the three EPS layers. The highest humic substance concentration was observed in soluble EPS (47.86 mg-C/L) of CNT supplemented group, while the concentration of HS was much lower in magnetite and control

groups regardless of EPS layers (Table 2). HS are widely reported as electron mediators facilitating long distance electron transfer between syntrophic microbes via its redox moiety - quinone [37, 38]. In this study, the significant high concentration of HS in soluble EPS of CNT groups could promote long distance electron transfer, thus accelerated methanogenesis. In fact, the electron transfer by HS and CNT follows the similar multi-hopping mechanism because of the same functional moiety-quinone [32]. This indicates that CNT and HS may complement each other to support microbial cell and EET.

Lovley et al. [38] reported that HS could stimulate iron-reducing bacteria for benzene-oxidizing, where soluble HS accelerated the reaction by alleviating direct contact as electron shuttles. Niedzwiecka et al. [39] also reported both AQDS (a model HS compound) and ferric ions can act as electron shuttle during 2,4-Dinitroanisole reduction. These facts indicate soluble HS and/or ferric ions can go through reversible redox reaction for stimulating EET between syntrophic partners in anaerobic biological system. In this study, three factors, namely, abundant HS (Table 2), high concentration of ferric ions (Table S2), and highly enriched iron reducing bacteria (illustrated in microbial community part), were in favor of establishing EET between syntrophic microorganisms in magnetite supplemented groups. It is hypothesized that the electron shuttles mediated EET performed in a two-step process in which iron reducing bacteria oxidized phenol with HS/ferric ions as electron acceptor, then the reduced shuttles transferred electron to terminal acceptors.

Therefore, other than the commonly reported EET pathway that CMs conduit electrons transfer between syntrophic partners directly, CMs also altered electron moieties compositions within EPS, i.e. HS and redox proteins, which could act as electron shuttles for long distance EET.

3.4 Shifts of microbial community diversity and structure

The DNA samples were sequenced using the Illumina Miseq platform. High Good's coverage (0.9985 to 0.9993) was observed in all samples indicating sufficient sequencing depth. Generally, the index of OTUs and Chao1 declined with the increase of FPC (Table S3). The topology of UPGMA tree clearly exhibited the similarity of microbial communities among all samples. As shown in Fig. 5, microbial community derived significantly with the increase of FPC, which indicates that FPC was an important factor in determining microbial structure. In general, the microbial community, which was fed with phenol only, can be divided into three clusters, (1) cluster at 200 mg/L FPC, (2) cluster at 400 mg/L to 600 mg/L FPC, and (3) cluster at 800 to 1000 mg/L FPC. The first separation of clusters could be induced by the change of substrate components, changing from co-substrate (glucose and phenol) to phenol. The separation of second and third clusters was corresponding to the decreased methane production and phenol degradation rate. The synchronous thresholds suggest the close correlation of microbial community structure and system performance.

The results also suggest that CMs were another important factor affecting microbial community evolution. Different materials showed diverged influence. The microbial community of CNT always clustered with control groups in all phenol fed only phases, whereas magnetite groups appeared to separate from them at all phases (Fig. 5). The results imply CNT imposed little influence on community structure, whereas magnetite nanoparticles could alter the community structure greatly. The great divergence between magnetite groups and CNT/control groups likely because magnetite can enrich iron reducing bacteria [40], which may induce extracellular iron redox electron transfer pathway. The results of microbial phylotype at phylum level also confirm this point (Fig. 6).

3.5 Microbial phylotypes alternation and correlation with CMs

3.5.1 Microbial community at phylum level

Fig. 6 shows the phylogenetic levels of phylum obtained from microbial community analysis. Similar to UPGMA tree result, all phylum of CNT and control groups show similar trend with increased FPC. At FPC 200 mg/L, the most abundant phylum was *Firmicutes* (control 50.3%, CNT 53.7%). And the highest abundant population shifted to *Proteobacteria* at FPC 400 mg/L (control 36.7 %, CNT 44.7%), and further to *Aminicenantes* and *Proteobacteria* beyond FPC 600 mg/L. *Firmicutes* are able to form spores to resist environmental stress, and quickly respond to substrate availability, thus they are able to outgrow other organisms [41]. *Proteobacteria* are gram-negative bacteria, in which many phenol degradation strains belong to [42]. The high abundance of *Proteobacteria* in this study suggests its important role in phenol degradation. *Aminicenantes* are commonly found in extreme environments [43], which were also found in this study when FPC was higher than 600 mg/L.

However, the most predominate phylum in magnetite groups at FPC of 200 mg/L, 400 mg/L, and >600 mg/L were *Bacteroidetes* (48.5%), *Actinobacteria* (48.8%), and *Proteobacteria/Actinobacteria* (around 30%), respectively. The abundance of *Bacteroidetes* could be triggered by the presence of ferric iron. According to Pan et al. [44], *Bacteroidetes* were enriched, while *Firmicutes* were weakened in an anaerobic dye degradation reactor, with the dose of nanoscale zero valent iron. Therefore, it is possible that the enrichment of *Bacteroidetes* were related with iron reducing activities. *Actinobacteria* were commonly found in active metal-reducing communities [45, 46], and its enrichment only happened in high concentration of carbon source impacted environment [47]. Therefore, *Actinobacteria*, selected by high FPC, may be responsible for iron reducing as well. Similar to control and CNT groups, the enrichment of

Proteobacteria was probably responsible for phenol degradation. However, the abundance of this phylum were remarkably different between magnetite and control/CNT groups. Compared with CNT group, the existence of magnetite nanoparticle had significantly altered the microbial structure. Except for *Proteobacteria*, the main enriched phylum in magnetite groups was predicted to be related to iron reducing bacteria, which could interact with methanogens via direct EET for stimulating electron transfer [6, 48].

3.5.2 Functional genera identification

The addition of conductive materials certainly enhanced the enrichment of phenol degradation bacteria (Fig. 7). Specifically, the total percentage of identified phenol degradation bacteria in CNT and magnetite groups were 24.4% and 33.8%, while only 13.9% was observed in control group (Fig. 7). However, the functional genera were quite different between the two CMs supplemented groups. The predominate functional genus was *Syntrophus* (12.3%) in CNT supplemented groups, whereas *Syntrophorhabdus* (14.1%) was the most abundant genus in magnetite supplemented group. In CNT supplemented group, *Brooklawnia* and *Treponema* constituted 3.5% and 3.4%, whereas their corresponding abundance were 9.3% and 0.9% in magnetite supplemented group. It is noteworthy that the electroactive bacteria- *Syntrophus* [49-51], exhibited around 6 times higher abundance in CMs supplemented groups compared to control groups.

Four genera of methanogens, i.e. *Methanosaeta*, *Methanolinea*, *Methanobacterium*, and *Methanoregula*, were observed. Fig. 8 shows the archaea community from genus level, which also exhibited great difference among control and CMs groups. The predominate archaea in CMs were *Methanosaeta* (around 40%), whereas *Methanobacterium* was the most abundant in control group (56.3%). Among the methanogens, only *Methanosaeta* and *Methanosarcina* were reported

with the capability of accepting electron directly[8, 52]. Similar to former magnetite amended anaerobic digestion [53], the high abundance of *Methanosaeta* in CMs groups was likely induced by the presence of CMs. *Methanobacterium* is a typical hydrogenotrophic methanogens that live exclusively through the reduction of carbon dioxide by hydrogen. The different methanogens in CMs and control groups indicates the change of methanogenesis from hydrogenotrophic to direct EET pathway. Thus, it is reasonable that both *Syntrophus* and *Methanosaeta* were essential in anaerobic phenol degradation via direct EET pathway.

4. Conclusions

The study demonstrated that the addition of CMs could improve anaerobic microbial activity in phenol degradation and enhance microbial resilience to adverse environmental impact. The observed phenomenon may be attributed to three factors. (1) CMs can act as electron conduit to facilitate direct EET; (2) CMs altered the EPS compositions, which were in favor of EET in both liquid phase and cell surface; (3) CMs enhanced the growth of functional genera in phenol degradation and direct electron transfer. It was found out that CNT and magnetite nanoparticles can trigger different microbial community to stimulate EET. Interestingly, the microbial community in control groups was not significantly different compared to carbon nanotube supplemented groups during the whole operation period, whereas the magnetite supplemented groups diverged largely from the initial stage.

References

- [1] F. Rosenkranz, L. Cabrol, M. Carballa, A. Donoso-Bravo, L. Cruz, G. Ruiz-Filippi, R. Chamy, J.M. Lema, Relationship between phenol degradation efficiency and microbial community structure in an anaerobic SBR, *Water Res.* 47 (2013) 6739-6749.
- [2] Z. Li, Y. Inoue, D. Suzuki, L. Ye, A. Katayama, Long-Term Anaerobic Mineralization of Pentachlorophenol in a Continuous-Flow System Using Only Lactate as an External Nutrient,

Environ. Sci. Technol. 47 (2013) 1534-1541.

[3] M.J. McInerney, J.R. Sieber, R.P. Gunsalus, Syntrophy in anaerobic global carbon cycles, *Curr. Opin. Biotechnol.* 20 (2009) 623-632.

[4] C.D. Dube, S.R. Guiot, Direct Interspecies Electron Transfer in Anaerobic Digestion: A Review, *Adv. Biochem. Eng. Biotechnol.* 151 (2015) 101-115.

[5] F. Liu, A.-E. Rotaru, P.M. Shrestha, N.S. Malvankar, K.P. Nevin, D.R. Lovley, Promoting direct interspecies electron transfer with activated carbon, *Energy Environ. Sci.* 5 (2012) 8982-8989.

[6] S. Kato, K. Hashimoto, K. Watanabe, Methanogenesis facilitated by electric syntrophy via (semi) conductive iron- oxide minerals, *Environ. Microbiol.* 14 (2012) 1646-1654.

[7] C. Cruz Viggi, S. Rossetti, S. Fazi, P. Paiano, M. Majone, F. Aulenta, Magnetite particles triggering a faster and more robust syntrophic pathway of methanogenic propionate degradation, *Environ. Sci. Technol.* 48 (2014) 7536-7543.

[8] Q. Yin, S. Yang, Z. Wang, L. Xing, G. Wu, Clarifying electron transfer and metagenomic analysis of microbial community in the methane production process with the addition of ferroferric oxide, *Chem. Eng. J.* 333 (2018) 216-225.

[9] Z. Zhao, Y. Zhang, Y. Li, Y. Dang, T. Zhu, X. Quan, Potentially shifting from interspecies hydrogen transfer to direct interspecies electron transfer for syntrophic metabolism to resist acidic impact with conductive carbon cloth, *Chem. Eng. J.* 313 (2017) 10-18.

[10] S. Kato, K. Hashimoto, K. Watanabe, Microbial interspecies electron transfer via electric currents through conductive minerals, *PANS* 109 (2012) 10042-10046.

[11] Y. Jing, J. Wan, I. Angelidaki, S. Zhang, G. Luo, iTRAQ quantitative proteomic analysis reveals the pathways for methanation of propionate facilitated by magnetite, *Water Res.* 108 (2016) 212-221.

[12] Y. Xiao, F. Zhao, Electrochemical roles of extracellular polymeric substances in biofilms, *Curr. Opin. in Electrochem.* 4 (2017) 206-211.

[13] H.C. Flemming, T.R. Neu, D.J. Wozniak, The EPS matrix: the "house of biofilm cells", *J. Bacteriol.* 189 (2007) 7945-7947.

[14] G.P. Sheng, H.Q. Yu, X.Y. Li, Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review, *Biotechnol. Adv.* 28 (2010) 882-894.

[15] L. Shi, H. Dong, G. Reguera, H. Beyenal, A. Lu, J. Liu, H.-Q. Yu, J. Fredrickson, Extracellular electron transfer mechanisms between microorganisms and minerals, *Nat. Rev. Microbiol.* 14 (2016) 651-662.

[16] F. Liu, A.-E. Rotaru, P.M. Shrestha, N.S. Malvankar, K.P. Nevin, D.R. Lovley, Promoting direct interspecies electron transfer with activated carbon, *Energy Environ. Sci.* 5 (2012) 8982-8989.

[17] W. Yan, N. Shen, Y. Xiao, Y. Chen, F. Sun, V. Kumar Tyagi, Y. Zhou, The role of conductive materials in the start-up period of thermophilic anaerobic system, *Bioresour. Technol.* 239 (2017) 336-344.

[18] Y.S. Kang, S. Risbud, J.F. Rabolt, P. Stroeve, Synthesis and characterization of nanometer-size Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ particles, *Chem. Mater.* 8 (1996) 2209-2211.

[19] APHA, Standard methods for the examination of water and wastewater, American Public Health Association (APHA): Washington, DC, USA (2005).

[20] A.P.H. Association, APHA Standard methods for the examination of water and wastewater, Am. Publ. Health Assoc. CD-ROM, 2005.

- [21] J. Yin, X. Tan, N. Ren, Y. Cui, L. Tang, Evaluation of heavy metal inhibition of activated sludge by TTC and INT-electron transport system activity tests, *Water Sci. Technol.* 52 (2005) 231-239.
- [22] M.H. Zwietering, I. Jongenburger, F.M. Rombouts, K. Van't Riet, Modeling of the bacterial growth curve, *Appl. Environ. Microbiol.* 56 (1990) 1875-1881.
- [23] W. Zhang, B. Cao, D. Wang, T. Ma, H. Xia, D. Yu, Influence of wastewater sludge treatment using combined peroxyacetic acid oxidation and inorganic coagulants re-flocculation on characteristics of extracellular polymeric substances (EPS), *Water Res.* 88 (2016) 728-739.
- [24] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric Method for Determination of Sugars and Related Substances, *Anal. Chem.* 28 (1956) 350-356.
- [25] B. Frolund, T. Griebe, P.H. Nielsen, Enzymatic activity in the activated-sludge floc matrix, *Appl. Microbiol. Biotechnol.* 43 (1995) 755-761.
- [26] T.T. Firozjaee, G.D. Najafpour, A. Asgari, Z. Bakhshi, R. Pishgar, N. Mousavi, Phenol biodegradation kinetics in an anaerobic batch reactor, *World Environmental and Water Resources Congress 2011: Bearing Knowledge for Sustainability*, 2011, pp. 4313-4321.
- [27] H. Movahedin, R. Shokoohi, A. Parvaresh, M. Hajia, A. J Jafari, Evaluating the effect of glucose on phenol removal efficiency and changing the dominant microorganisms in the serial combined biological system, *J. Res. Health. Sci.* 4 (2011) 8-13.
- [28] F.E. Jenney, M.F. Verhagen, X. Cui, M.W. Adams, Anaerobic microbes: oxygen detoxification without superoxide dismutase, *Science* 286 (1999) 306-309.
- [29] A.F. Salvador, G. Martins, M. Melle- Franco, R. Serpa, A.J. Stams, A.J. Cavaleiro, M.A. Pereira, M.M. Alves, Carbon nanotubes accelerate methane production in pure cultures of methanogens and in a syntrophic coculture, *Environ. Microbiol.* 19 (2017) 2727-2739.
- [30] Y. Liu, Y. Zhang, X. Quan, S. Chen, H. Zhao, Applying an electric field in a built-in zero valent iron – Anaerobic reactor for enhancement of sludge granulation, *Water Res.* 45 (2011) 1258-1266.
- [31] X. Liu, J. Chen, Y. Huang, X. Sun, G. Sheng, D. Li, L. Xiong, Y. Zhang, F. Zhao, H. Yu, Experimental and theoretical demonstrations for the mechanism behind enhanced microbial electron transfer by CNT network, *Sci. Rep.* 4 (2014) 3732.
- [32] D.R. Lovley, Happy together: microbial communities that hook up to swap electrons, *The ISME J.* 11 (2017) 327-336.
- [33] Y. Xiao, F. Zhao, Electrochemical roles of extracellular polymeric substances in biofilms, *Current Opinion in Electrochemistry* 4 (2017) 206-211.
- [34] Y. Jing, J. Wan, I. Angelidaki, S. Zhang, G. Luo, iTRAQ quantitative proteomic analysis reveals the pathways for methanation of propionate facilitated by magnetite, *Water Res.* 108 (2017) 212-221.
- [35] L. Ren, D. Yan, W. Zhong, Enhanced enzyme activity through electron transfer between single-walled carbon nanotubes and horseradish peroxidase, *Carbon* 50 (2012) 1303-1310.
- [36] B. Cao, L. Shi, R. Brown, Y. Xiong, J. Fredrickson, M. Romine, M. Marshall, M. Lipton, H. Beyenal, Extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics, *Environ. Microbiol.* 13 (2011) 1018-1031.
- [37] E. Roden, A. Kappler, I. Bauer, J. Jiang, A. Paul, R. Stoesser, H. Konishi, H. Xu, Extracellular electron transfer through microbial reduction of solid-phase humic substances, *Nature Geoscience* 3 (2010) 417-421.
- [38] D. Lovley, J. Coates, E. Blunt Harris, E.J.P. Phillips, J. Woodward, Humic substances as

electron acceptors for microbial respiration, *Nature* 382 (1996) 445-448.

[39] J.B. Niedzwiecka, S.R. Drew, M.A. Schlautman, K.A. Millerick, E. Grubbs, N. Tharayil, K.T. Finneran, Iron and Electron Shuttle Mediated (Bio)degradation of 2,4-Dinitroanisole (DNAN), *Environ. Sci. Technol.* 51 (2017) 10729-10735.

[40] Z. Yang, R. Guo, X. Shi, C. Wang, L. Wang, M. Dai, Magnetite nanoparticles enable a rapid conversion of volatile fatty acids to methane, *RSC Adv.* 6 (2016) 25662-25668.

[41] M. Schaechter, *Encyclopedia of microbiology*, Academic Press 2009.

[42] Y. Jiang, L. Wei, H. Zhang, K. Yang, H. Wang, Removal performance and microbial communities in a sequencing batch reactor treating hypersaline phenol-laden wastewater, *Bioresour. Technol.* 218 (2016) 146-152.

[43] I.F. Farag, J.P. Davis, N.H. Youssef, M.S. Elshahed, Global patterns of abundance, diversity and community structure of the Aminicenantes (candidate phylum OP8), *PLoS One* 9 (2014) e92139.

[44] F. Pan, X. Zhong, D. Xia, X. Yin, F. Li, D. Zhao, H. Ji, W. Liu, Nanoscale zero-valent iron/persulfate enhanced upflow anaerobic sludge blanket reactor for dye removal: Insight into microbial metabolism and microbial community, *Sci. Rep.* 7 (2017) 44626.

[45] B. Lin, C. Hyacinthe, S. Bonneville, M. Braster, P. Van Cappellen, W.F. Röling, Phylogenetic and physiological diversity of dissimilatory ferric iron reducers in sediments of the polluted Scheldt estuary, Northwest Europe, *Environ. Microbiol.* 9 (2007) 1956-1968.

[46] X. Wang, J. Yang, X. Chen, G. Sun, Y. Zhu, Phylogenetic diversity of dissimilatory ferric iron reducers in paddy soil of Hunan, South China, *J. Soils Sed.* 9 (2009) 568-577.

[47] D.M. Akob, L. Kerkhof, K. Kusel, D.B. Watson, A.V. Palumbo, J.E. Kostka, Linking specific heterotrophic bacterial populations to bioreduction of uranium and nitrate in contaminated subsurface sediments by using stable isotope probing, *Appl. Environ. Microbiol.* 77 (2011) 8197-8200.

[48] M. Morita, N.S. Malvankar, A.E. Franks, Z.M. Summers, L. Giloteaux, A.E. Rotaru, C. Rotaru, D.R. Lovley, Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates, *J. Microbiol.* 2 (2011) e00159-00111.

[49] I.V. Khilyas, A.A. Sorokin, L. Kiseleva, D.J. Simpson, V. Fedorovich, M.R. Sharipova, M. Kainuma, M.F. Cohen, I. Goryanin, Comparative Metagenomic Analysis of Electrogenic Microbial Communities in Differentially Inoculated Swine Wastewater-Fed Microbial Fuel Cells, *Scientifica* 2017 (2017).

[50] C. Reimers, P. Girguis, H. Stecher, L. Tender, N. Ryckelynck, P. Whaling, Microbial fuel cell energy from an ocean cold seep, *Geobiology* 4 (2006) 123-136.

[51] R. Toczyłowska-Mamińska, K. Szymona, M. Kloch, Bioelectricity production from wood hydrothermal-treatment wastewater: Enhanced power generation in MFC-fed mixed wastewaters, *Sci. Total Environ.* 634 (2018) 586-594.

[52] A.E. Rotaru, P.M. Shrestha, F. Liu, B. Markovaite, S. Chen, K.P. Nevin, D.R. Lovley, Direct interspecies electron transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*, *Appl. Environ. Microbiol.* 80 (2014) 4599-4605.

[53] Y. Lei, L. Wei, T. Liu, Y. Xiao, Y. Dang, D. Sun, D.E. Holmes, Magnetite enhances anaerobic digestion and methanogenesis of fresh leachate from a municipal solid waste incineration plant, *Chem. Eng. J.* 348 (2018) 992-999.

Fig. 1. Phenol degradation profiles in each phase. (a): Phase 1; (b): Phase 2; (c): Phase 3; (d): Phase 4; (e): Phase 5; (f): Phase 6.

C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Fig. 2. Methane production profiles in each phase. (a): Phase 1; (b): Phase 2; (c): Phase 3; (d): Phase 4; (e): Phase 5; (f): Phase 6.

C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Fig. 3. Maximum CH_4 production rate (R_{max}) at begin (B), middle (M) and final (F) feeding cycles of all phases.

P1- P6 are the abbreviation of phase 1 to phase 6.

C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Fig. 4. Electron transport system activity in phase 6.

C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Fig. 5. UPGMA cluster based-on 16s RNA of community samples.

P2-P6 are the abbreviation of phase2 to phase 6.

C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Fig. 6. Relative abundances of phylogenetic groups in source sludge, control group, CNT supplemented groups and magnetite supplemented groups. Microbial communities are presented in taxonomic phylum. Phylogenetic groups accounting for ≤ 2.0 % of all classified sequences are summarized in the artificial group “others”. P2- P6 are the abbreviation of phase2 to phase 6.

C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Fig. 7. Relative abundance of identified phenol degradation bacteria in phase 6.
C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Fig. 8. Relative abundance of archaea in phase 6.
 C-Control group; M-Magnetite nanoparticles supplemented group; CNT-Carbon nanotube supplemented group.

Table 1 Operation conditions, phenol degradation performance monitoring and microbial community sampling time.

	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6
Duration (Day)	1-15	15-45	45-75	75-105	105-135	135-165
First feeding cycle (day)	1-4	15-17	45-47	75-81	105-112	135-144
Middle feeding cycle (day)	7-10	31-33	61-63	87-93	120-127	144-154
Last feeding cycle (day)	13-15	43-45	72-75	99-105	127-135	154-165
Glucose (mg/L)	138	\	\	\	\	\
Phenol (mg/L)	138	200	400	600	800	1000
Total COD (mg/L)	476.1	476	952	1428	1904	2380
DNA extraction (day)	15	45	75	105	135	165

Table 2 Quantification of sub-fractions (mg-C/L) of observed organic compounds in EPS.

Sample	Hydrophobic DOC	HMW protein	LMW protein	HMW Carbohydrate	LMW Carbohydrate	Humic substances	Building blocks	LMW Neutrals	LMW Acids
SB-C	24.70	7.54	12.27	2.98	5.59	8.12	4.89	19.36	0.54
SB-M	43.58	4.67	234.82	7.25	1.16	4.94	3.65	4.28	0.00
SB- CNT	20.46	6.92	105.77	9.18	1.45	25.20	22.66	10.03	0.00
LB-C	2.15	1.27	7.12	0.60	0.95	1.10	1.16	4.86	0.35
LB-M	0.21	1.92	24.91	1.70	0.43	3.02	4.52	4.54	1.05
LB- CNT	0.15	1.64	18.85	0.58	1.19	1.39	2.19	5.16	1.22
TB-C	5.71	1.45	13.95	0.54	0.84	0.63	2.52	8.09	0.60
TB-M	0.82	2.41	24.25	2.13	1.35	1.71	3.56	8.45	0.46
TB- CNT	0.52	0.16	29.25	0.17	1.35	0.24	0.53	1.38	0.00

DOC: Hydrophobic dissolved organic carbon; HMW: High molecular weight; LMW: Low molecular weight; SB-C/SB-M/SB-CNT: Soluble EPS in control group/ magnetite nanoparticles supplemented group/ CNT supplemented group. LB-C/LB-M/LB-CNT: Loosely bound EPS in control group/ magnetite nanoparticles supplemented group/ CNT supplemented group; TB-C/TB-M/TB-CNT: Tightly bound EPS in control group/ magnetite nanoparticles supplemented group/ CNT supplemented group;