Response of Anaerobic Granular Sludge to Iron Oxide Nanoparticles and
Multi-Wall Carbon Nanotubes during Beet Sugar Industrial Wastewater
Treatment

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Abstract

The accelerated use of iron oxide nanoparticles (IONPs) and multi-wall carbon nanotubes (MWCNTs) in the consumer and industrial sectors has triggered the need to understand their potential environmental impact. The response of anaerobic granular sludge (AGS) to IONPs and MWCNTs during the anaerobic digestion of beet sugar industrial wastewater (BSIW) was investigated in this study. The IONPs increased the biogas and subsequent CH₄ production rates in comparison with MWCNTs and the control samples. This might be due to the utilization of IONPs and MWCNTs as conduits for electron transfer toward methanogens. The MWCNTs majorly enriched the bacterial growth, while IONP enrichment mostly benefitted the archaea population. Furthermore, scanning electron microscopy and confocal laser scanning microscopy revealed that AGS produced extracellular polymeric substances, which interacted with the IONPs and MWCNTs. This provided cell protection and prevented the nanoparticles from piercing through the membranes and thus cytotoxicity. The results provide useful information and insights on the adjustment of anaerobic microorganisms to the natural complex environment based on nanoparticles infiltration.

Keywords: anaerobic granular sludge, extracellular polymeric substance, iron oxide nanoparticle, multi-wall carbon nanotube, toxicity

1. Introduction

The use of nanomaterials in consumer and industrial products has accelerated the development and application of nanotechnology. However, its integration into various sectors, including pharmaceutical, energy, agriculture, construction, and wastewater treatment (Yadav et al. 2016, Casals et al. 2014), has raised concerns with respect to the environmental safety; most investigations focus on the potential effects on human health and the environment (Helland et al. 2008, Petersen et al. 2011). Some studies have reported the toxic effects of carbon nanotubes (Yadav et al. 2016, Mwangi et al. 2012, Simon-Deckers et al. 2009) and zinc oxide nanoparticles (Mu et al. 2012) on microorganisms, cells, and bacteria.

Because the microbial community plays a pivotal role in the conversion of organic matter to methane and carbon dioxide, both in the natural environment and engineered systems (Li et al. 2015), it is crucial to examine their interaction with nanoparticles. For instance, the consumption of electrons during the reduction of carbon dioxide to methane by methanogens, which function as a sink, is a very important process; yet, it is too delicate due to their hypersensitivity to heavy metals and nanoparticles (Mu et al. 2012, Chen et al. 2008). Therefore, understanding the effectiveness of the

interelectron transfer process in the syntrophic microbial community during the degradation of organic matter is an essential step of the beet sugar industrial wastewater (BSIW) treatment due to its high potential of methane gas generation (Angelidaki and Sanders 2004).

For a very long time, organic matter degradation was known to be accomplished through interspecies H_2 (or formate) transfer between H_2 -producing acetogenic bacteria and H_2 -consuming methanogenic archaea (Stams et al. 2006, Stams and Plugge 2009). However, its success majorly depended on the maintenance of low concentrations of H_2 by methanogens. This method became unpopular due to the lack of effective techniques for the determination of turnover rates of H_2 and formate. Recent studies revealed that microorganisms are capable of directly exchanging electrons (apart from H_2 or formate transfer) through a process known as direct interelectron transfer (DIET; (Lovley 2011, Malvankar and Lovley 2012, Shrestha et al. 2013). For example, *Geobacter* strains (*Geobacter metallireducens* and *Geobacter sulfurreducens*) are capable of DIET via conductive appendages (pili) and/or redox protein (cytochromes) in an ethanol-fed co-culture (Summers et al. 2010, Nagarajan et al. 2013). In addition, *Geobacter metallireducens* provides a direct pathway for electron exchange with methanogens (*Methanosaeta* species; (Rotaru et al.

2014). Furthermore, studies have revealed that electrically conductive material can be utilized as conduits during the DIET process between electron-donating and electron-accepting microbes. This pathway has been established with single wall carbon nanotubes (Li et al. 2015), activated carbon (Liu et al. 2012), and minerals such as magnetite (Kato et al. 2012a, b, Cruz Viggi et al. 2014). Thus, the addition of IONPs and MWCNTs might represent a novel and still unexplored strategy to increase the conversion rate of organic matter into methane gas.

Therefore, the aim of this study was to investigate the response of anaerobic reduction of BSIW in the presence of IONPs and MWCNTs using an expanded granular sludge bed reactor (EGSB). We demonstrate that the addition of IONPs and MWCNTs triggers DIET, which subsequently promotes the conversion of BSIW into methane. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were used to visualize the effect of extracellular polymeric substances (EPS) on anaerobic granular sludge (AGS). The effects of IONPs and MWCNTs on the microbial consortia and chemical oxygen demand (COD) removal efficiency were also investigated.

2. Materials and methods

2.1 Configuration and operation procedure of anaerobic reactors

Three Plexiglas EGSB reactors (1.2-L volume, 4-cm diameter, and 56-cm height) were utilized in this experiment. Each reactor has a working volume of 0.9 L and consists of a temperature controller and online pH and oxidation–reduction potential (ORP) detectors. Peristaltic pumps (BT100-2J, Longer Pump, China) were used for the feeding and recycling at controlled flow rates. An influent was introduced into the reactor in an up-flow mode (Fig. 1).

[Figure 1 here]

The system was maintained at mesophilic conditions at a temperature of $36.0^{\circ}\text{C} \pm 1^{\circ}\text{C}$ using a temperature controller. It was operated at different hydraulic retention times (HRTs) gradually decreasing from 48 to 12 h. The pH was regulated at 6.9 ± 0.2 using a buffer (sodium hydrogen carbonate). The evolved biogas was collected using a gasbag, measured, recorded, and normalized to negate reactor head space effects. A normalization was carried

out as reported. The ORP, temperature, and pH were observed daily, while COD, volatile fatty acids (VFA), and biogas were measured and recorded every two days. Each of the reactors was seeded with granular sludge (0.879 g volatile-suspended solids, VSS/L) to 44.4% (v/v) of its working volume. The seed AGS, which was used as inoculum, was obtained from the beer industry (latitude 45°49'N, longitude 126°42'E) in Harbin in the Heilongjiang Province, China. A 400-mL volume of sludge was fed into each reactor after mixing with nanomaterials (there was no nanomaterial in the control reactor).

2.2 Nanomaterial preparation

Commercially accessible IONPs and MWCNTs were obtained from the Alpha Nano Technology Company (Chengdu, China). The factory characteristics of the IONPs and MWCNTs were a purity of 99.9% and 97.99% and outer diameter (OD) of 20 nm and 10–20 nm, respectively. The stock suspensions for the nanomaterials were mixed with deionized water and ultrasonicated for 1 h to break the aggregates using a sonicator (VCX130-USA) at a power output of 130 W and 20 KHz. Subsequently, they were centrifuged and dried before measuring the IONPs of the 750-mg/L and MWCNTs of the 1500-mg/L concentrations.

2.3 Medium composition and characteristics

The three EGSB reactors for the IONPs, MWCNTs, and control sample were fed with beet sugar industrial wastewater for 74 days, which was collected from the sugar beet factory in Nehe in the Heilongjiang Province, China. Its characteristics are listed in Table 1.

[Table 1 here]

2.4 Confocal laser scanning microscopy and scanning electron microscopy of anaerobic granular sludge

The collected anaerobic granules were fixed with 2.5% glutaraldehyde in phosphate-buffered saline after the exposure to IONPs and MWCNTs, followed by fluorescent staining with FITC, Con A, and calcofluor-white for protein and α -D-glucopyranose polysaccharides and β -D-glucopyranose polysaccharides, respectively. This procedure was carried out as outlined in Li et al. (2015). The collected samples were pretreated for SEM analysis

using energy dispersive spectroscopy (EDS) following the procedure outlined by Jin et al. (2007).

2.5 Microbial community analysis

After 74 days of exposure of the sludge granules to IONPs and MWCNTs, the sludge samples were collected and the microbial community composition was analyzed by pyrosequencing. Agarose gel was run to check the integrity and the concentration of the extracted genomic deoxyribonucleic acid (DNA) and quantified using the Qubit 2.0 DNA kit for the polymerase chain reaction (PCR) reaction. The fused universal primer of the MiSeq sequencing platform was used for the PCR primer. Subsequently, agarose gel electrophoresis was performed to test the PCR products and recover the DNA. The process was conducted based on the Sangon agarose recovery kit from Sangon Biotech Co., Ltd. (Shanghai, China). The obtained sequences were phylogenetically assigned to the domain phylum, class, order, family, and genus based on the Bergeys taxonomic group using a Ribosomal Database Project naïve Bayesian ribosomal DNA classifier with a confidence threshold of 80%. Closest matching nucleotide sequences in the GenBank database searches were performed using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>). The

partial archaeal and bacterial 16S rRNA gene sequences determined in this study were then deposited in the Genbank under the following accession numbers: KX926559, KX926560, KX926562, KX926564 KX926565, KY346968, and KY346969.

3. Results

3.1 Substrate utilization in anaerobic digestion

Figure 2 shows the COD removal efficiency of beet sugar industrial wastewater with an influent concentration of 3000 mg/L, without nanoparticles (control sample) for MWCNTs and IONPs in 74 days. During the initial three days of the experiment with a HRT of 48 h, the COD concentration rapidly decreased in the control sample reactor compared with that of the MWCNT and IONP reactors, resulting in an effluent concentration of 738, 837, and 1089 mg/L, respectively. This can be attributed to the time taken by the microorganisms to acclimatize due to the change of the environmental conditions as a result of the exposure to nanoparticles as opposed to the control reactor. After 18 days of operation, the IONP reactor had the highest COD removal efficiency of 95%; the MWCNT and control reactors both showed an efficiency of 92%. After 22 days, the HRT of the reactors was

changed to 24 h. The COD removal efficiency after 48 days was 95%, 94%, and 89% for IONPs, control sample, and MWCNTs, respectively. The HRT was then changed to 12 h. During this period, the IONP and MWCNT reactors exhibited a higher COD removal efficiency than the control reactor. Although the MWCNT reactor often had the lowest COD removal efficiency, its performance increased steadily; it obtained the highest rates after 72 days of operation. This resulted in COD concentrations of 142, 154, and 197 mg/L for the MWCNT, IONP, and control reactors after 74 days, respectively.

[Figure 2 here]

3.2 Biogas and CH₄ production

The methane gas production in the anaerobic digestion is one of the indicators of a successful process. Figure 3 shows the apparent influences of IONPs and MWCNTs on the biogas (a), cumulative biogas (b), and cumulative methane production (c). During the incubation period of 74 days, no inhibitory effects were observed at the dosages of 1500 mg/L MWCNTs and 750 mg/L IONPs. The gas composition was determined from the reactor headspace after a

regular time interval of two days. The initial stages of the anaerobic digestion of BSIW were characterized by the emission quantity of the scintilla of biogas (Fig. 3a).

In the first 24 days, the IONP reactor produced cumulatively more biogas than the control reactor, while the control reactor production was higher than that of the MWCNT reactor (Fig. 3b). This resulted in 485.7, 395.2, and 370.5 ml/g VSS of biogas production of the IONP, control, and MWCNT reactors, respectively. This trend changed after 38 days, the IONP, MWCNT, and control reactors produced 1857.0, 1345.6, and 1030.4 ml/g VSS of cumulative biogas, respectively. Although the highest quantity of biogas was produced in the IONP reactor (Fig. 3a), the cumulative CH₄ gas content was the highest in the MWCNT reactor (Fig. 3c). The MWCNT, IONP, and control reactors generated 65.3, 62.0, and 18.5 ml/g VSS of CH₄, respectively. Subsequently, the IONPs induced more biogas and CH₄ production. The IONP, MWCNT, and control reactors produced 25144.4, 21876.0, and 20082.8 ml/g VSS of biogas and 8374.9, 7313.2, and 6496.5 ml/g-VSS of CH₄, respectively. The IONPs induced more biogas production than the MWCNTs and control reactor (Fig. 3a). After the incubation period, the MWCNTs and IONPs generated 12.6% and 28.9% more ml/g-VSS CH₄ gas than the control reactor, respectively.

[Figure 3 here]

3.3 Hydrogen and volatile fatty acids changing profiles

Figure 4 shows the hydrogen (H_2) and volatile fatty acids (VFAs) as the main intermediate products during CH_4 production; their concentrations changed over the study period. Apart from the initial stage of anaerobic digestion during which the hydrogen content was higher (7.3%, 5.5%, and 2.8% for IONPs, control sample, and MWCNTs respectively), the content remained below 0.7% and even dropped below 0.4% after 60 days of operation (Fig. 4a). The production trend showed no significant difference; however, less H_2 was produced in the reactors with IONPs, which might be attributed to the rapid consumption of H_2 during CH_4 production, followed by the MWCNTs and control reactor with a slightly higher production. The analysis of the VFAs showed that the main products in aqueous solution were acetate and propionic acid. The initial stage of operation was characterized by an irregular trend of increase in the acetic and propionic acid concentrations before their production started to decline after ~20 days (Figs 4b and c). Subsequently, rapid consumption of VFAs was realized in the reactor with IONPs, followed by the MWCNTs

and control sample. After 50 days of anaerobic digestion, hardly any acetate acid production was observed in the IONP reactor and very little in the MWCNT reactor. The same was true for the propionic acid production, although the MWCNTs produced higher concentrations than the control sample. This indicates an agreement between the H_2 , VFA, and CH_4 production.

[Figure 4 here]

3.4 Chemical oxygen demand balance

The calculation of the COD balance was carried out to evaluate the removal data by considering the influent and effluent concentrations, which also include the methane production as COD removal. The parameters obtained when the reactors produced the highest biogas were used for the calculation. The calculation followed the procedure given in (Alkaya and Demirer 2011); the theoretical equivalence of methane was 35°C at 1 atm (2.53 g/L methane (Speece 1996). The highest biogas production of the IONPs and the MWCNTs and control sample was realized on days 62 and 66, respectively. The input COD (3.2 g) was compared with the output COD (2.7, 2.8, and 3.0 g), which accounted for 84.0%, 86.8%, and 93.0% for the MWCNTs, control sample, and IONPs, respectively. Although all COD inputs could not be accounted for,

the data is sufficiently reliable based on the variation. The carbon that could not be accounted for might have been converted to biomass due to microbial consortia growth.

3.5 Microbial community analysis

Microbial community analysis was carried out to investigate the structure of the anaerobic sludge exposed to IONPs and MWCNTs. Figure 5 shows the phylogenetic levels of phylum, class, order, and genus obtained from microbial community analysis. The microbial structures showed *Euryarchaeota*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Chloroflexi* in the order of the most predominant phylum to the least (Fig. 5a). It is evident that the archaeal phylum *Euryarchaeota* (63.0%) was highly enriched by IONPs, while *Bacteroidetes* (11.2%) and *Firmicutes* (8.4%) phylum were more enriched by MWCNTs. The microbes of the phylum *Crenarchaeota* drastically reduced in the presence of IONPs. At the class level (Fig. 5b), *Methanomicrobia* and *Anaerolineae* were greatly enriched in the presence of both IONPs and MWCNTs, while *Deltaproteobacteria*, *Thermoprotei*, and *Thermoplasmata* were significantly reduced. In addition, IONPs enriched *Methanobacteria*, while MWCNTs enriched *Bacteroidia*. This trend was also observed for the phylotype order (Fig. 5c). At

the genus level (Fig. 5d), *Methanosaeta*, *Methanobacterium*, *Methanosphaera*, and *Methanolinea* were highly enriched by IONPs, while *Methanospirillum*, *Parabacteroides*, *Methanomethylovorans*, *Longilinea*, and *Pyrolobus* equally improved in the presence of MWCNTs. *Thermogymnomonas*, *Desulfovibrio*, and *Paludibacter* were reduced when exposed to IONPs and MWCNTs.

The majority of the bacterial sequences of the *Longilinea* genera belongs to *Longilinea arvoryzae* species (accession number KX926564, 98% identity) and those of *Parabacteroides* were identified as *Parabacteroides chartae* species (accession number KX926565, 97% identity). The sequences of *Methanosaeta* genera conform to *Methanosaeta harundinacea* (accession number KX926560, 98% identity) species, while *Methanobacterium* genera belong to *Methanobacterium beijingense* species (accession number KX926562, 98% identity) and *Methanospirillum* genera sequences were identical to *Methanospirillum hungatei* species (accession number KX926559, 98% identity). The *Geobacter* species was represented at the genus level; its population has a 0.4%, 0.5%, and 0.5% representation in the IONP, MWCNT, and control reactors, respectively. The species conformed to *Geobacter lovleyi* sz (accession number KY346968 with 97% identity) and

Geobacter thiogenes (accession number KY346969 with 97% identity).

[Figure 5 here]

3.6 Microscopic imaging of anaerobic granular sludge

The sludge granules from the control, IONP, and MWCNT reactors were collected after the experiment and observed with SEM. Figure 6 displays the images of different structural features. Sludge from the control reactor (Fig. 6a) shows bacteria in form of dispersed loose aggregates, while IONP (Fig. 6b) and MWCNT (Fig. 6c) sludge granules appear to be more densely packed. The later phenomenon can be attributed to the interactions of the nanoparticles (IONPs and MWCNTs), microbial consortia, and their induced EPS. The microorganisms excrete EPS substances that attach themselves to the cells and provide a formidable protective defense against any interference from the external environment (Sheng et al. 2010). The EDS of the same samples showed peaks due to various metals (Figs 6d–f) in the granular sludge. The carbon content by weight was 53.53%, 50.49%, and 66.26%, while that of iron was 8.40%, 17.45%, and 2.41% in the

control (Fig. 6d), IONP (Fig. 6e) and MWCNT (Fig. 6f) reactors, respectively. The predominance of carbon and iron in the reactors with MWCNTs and IONPs in comparison with the control sample shows that the MWCNTs and IONPs were still available in the AGS of the reactors.

[Figure 6 here]

Further examination of the EPS distribution in the granular sludge was carried out by CLSM. Figure 7 shows the spatial distribution of the proteins and polysaccharides. The proteins, α -D-, and β -D-glucopyranose polysaccharides in the control reactor formed an evenly distributed pattern together with the structure of the sludge granules (a1–a3). On the other hand, proteins, α -D-, and β -D-glucopyranose polysaccharides formed layers around IONP (b1–b3) and MWCNT (c1–c3) sludge granules. This might be a defensive mechanism of the microbes against granule-internal intrusion by the nanoparticles.

[Figure 7 here]

4. Discussion

The sensitivity of the microorganisms to toxicants and the adverse effects on their catabolic activities are of concern; methanogens are in more danger because of their supersensitivity because they interact with nanoparticles such as carbon nanotubes (Mu et al. 2012). On the other hand, the use of nanoparticles can increase the microbial metabolism and lead to higher wastewater treatment levels and biomethanation potentials. It is anticipated that the application of IONPs and MWCNTs in the anaerobic digestion of beet sugar industrial wastewater (BSIW) would have either inhibitory effects or promote degradation and biomethanation. This hypothesis was tested by examining the response of AGS to 750 mg/L IONPs and 1500 mg/L MWCNTs. The results neither showed a significant difference in the COD removal efficiency nor adverse effects on the microbial activity but rather an explicitly increase in the biogas and, subsequently, biomethane production with the addition of IONPs and MWCNTs.

The toxicity to microorganisms is known to emanate from oxidative stress, residual metal catalysts, or direct membrane agitation (Kang et al. 2007, Pacurari et al. 2008, Pasquini et al. 2013). Because this study was conducted under anaerobic conditions, oxidative stress caused by reactive oxygen species generated by nanoparticles under aerobic conditions is not a possibility to trigger the toxicity. In addition, the reactors

continually operated at $\text{ORP} > -470 \text{ mV}$. On the other hand, metallic impurities could be toxic to microorganisms, even though reports indicate that 40% to 60% pure single-walled carbon nanotubes are non-toxic (Tong et al. 2012). In this study, IONPs and MWCNTs with a purity $> 97\%$ were utilized; hence, no impurities can be released and if any, then in scintilla quantity to catalyze toxicity. The cytotoxicity in this study might be due to direct membrane perturbation. However, the SEM and CLSM observations showed that the bacteria and EPS in the reactors were densely packed with the nanoparticles, preventing the nanoparticles from piercing through the membrane and averting cytotoxicity.

Biomethanation is a complex process accomplished through catabolic interactions involving different microorganisms such as fermenting bacteria, homoacetogenic bacteria, and methanogenic archaea (Schink 1997). These syntrophic partnerships utilize broken-down compounds, which are impossible to be digested by themselves (Stams 1994), supported by interspecies electron transfer (IET) from secondary fermenting bacteria to methanogenic archaea. This process is known to occur via diffusion of electron carriers such as hydrogen and formate in aqueous phases (Stams 1994, Ishii et al. 2005). In addition,

bacteria are also known to utilize conductive mineral particles as conduits for electron transfer (Kato et al. 2010).

The results of this study reveal that the interaction between AGS and IONPs and MWCNTs leads to higher biogas and CH₄ production rates due to bacteria substrate fermentation into VFAs and further conversion to CH₄ by methanogens. The predominance of the *Bacteroidetes* and *Firmicutes* bacterial phyla is an indicator for a successful fermentation process. For instance *Clostridium* bacteria, which belong to *Firmicutes*, are known to be active in VFA fermentation (Jiang et al. 2011). Also, the enrichment of *Longilinea arvoryzae*, a species known for its hydrolytic fermentation functionality (Lynd et al. 2002), might have accelerated the cellulosome degradation, releasing compounds that can be utilized by methanogens. On the other hand, the abundance of the acetotrophic methanogen *Methanosaeta* (39.4, 26.5, and 24.6 in IONPs, MWCNTs, and control, respectively, at the genus level) is consistent with low acetate concentrations in varied reactors. This might have increased the bioconversion of acetic acid to methane gas, resulting in more biogas and CH₄ production in the IONP and MWCNT reactors.

Studies have also indicated that DIET might occur in the presence of the *Geobacter* and *Methanosaeta* species (MallaáShrestha 2014). However, the *Geobacter* species

abundance is similar in all the reactors and the species is unknown to promote DIET. It is therefore possible that the *Geobacter* species barely made any direct electrical connection with the *Methanosaeta* species; hence, there might have been no DIET. It is therefore most probable that IONPs and MWCNTs were utilized as conduits for electrons transfer towards methanogens. This might have accelerated the DIET, leading to higher biogas production rates in comparison with the control reactor, which lacked conductive mineral particles as conduits to accelerate the electron transfer, despite of high COD removal efficiency, leading to low biogas production rates. It can further be hypothesized that the enrichment of methanogens (*Methanosaeta* species) by IONPs might have subsequently increased the biogas and methane production rates compared with the MWCNTs.

We observed higher biogas and methane production rates accompanied by a faster acetate and propionate utilization rate and lower H₂ accumulation in the 12-h HRT in the reactors with IONPs and MWCNTs. It is likely that the IONPs and MWCNTs acted as conduits for electron transfer together with anaerobes, facilitating DIET when mixed with AGS. The IONPs might have created more unique interspecies interactions than the MWCNTs. Further investigations are required.

5. Conclusions

The response of anaerobic granular sludge to 750 mg/L IONPs and 1500 mg/L MWCNTs in continuously fed EGSB reactors was investigated in this study. Although the substrate utilization showed no significant difference, MWCNTs and IONPs showed higher COD removal efficiencies than the control sample towards the end of the 12-h HRT. The IONPs lead to higher biogas and CH₄ generation, followed by the MWCNT and control reactors. The IONPs and MWCNTs might have acted as conduits between bacteria and methanogens during DIET. The investigation of AGS by SEM and CLSM revealed the production of EPS, which interacted with IONPs and MWCNTs, providing protection to the cells from external influences, thereby successfully averting cytotoxicity. The IONPs greatly enriched the microbial archaea community, while the MWCNTs enriched the bacterial composition. Further studies are necessary to fully understand the synergistic effect of IONPs and MWCNTs.

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References

- Yadav, T., Mungray, A.A. and Mungray, A.K. (2016) Effect of multiwalled carbon nanotubes on UASB microbial consortium. *Environmental Science and Pollution Research* 23(5), 4063-4072.
- Casals, E., Barrena, R., García, A., González, E., Delgado, L., Busquets- Fité, M., Font, X., Arbiol, J., Glatzel, P. and Kвашnina, K. (2014) Programmed iron oxide nanoparticles disintegration in anaerobic digesters boosts biogas production. *Small* 10(14), 2801-2808.
- Helland, A., Wick, P., Koehler, A., Schmid, K. and Som, C. (2008) Reviewing the environmental and human health knowledge base of carbon nanotubes. *Ciência & Saúde Coletiva* 13(2), 441-452.
- Petersen, E.J., Zhang, L., Mattison, N.T., O'Carroll, D.M., Whelton, A.J., Uddin, N., Nguyen, T., Huang, Q., Henry, T.B. and

Holbrook, R.D. (2011) Potential release pathways, environmental fate, and ecological risks of carbon nanotubes. *Environmental science & technology* 45(23), 9837-9856.

Mwangi, J.N., Wang, N., Ingersoll, C.G., Hardesty, D.K., Brunson, E.L., Li, H. and Deng, B. (2012) Toxicity of carbon nanotubes to freshwater aquatic invertebrates. *Environmental Toxicology and Chemistry* 31(8), 1823-1830.

Simon-Deckers, A., Loo, S., Mayne-L'hermite, M., Herlin-Boime, N., Menguy, N., Reynaud, C., Gouget, B. and Carrière, M. (2009) Size-, composition-and shape-dependent toxicological impact of metal oxide nanoparticles and carbon nanotubes toward bacteria. *Environmental science & technology* 43(21), 8423-8429.

Mu, H., Zheng, X., Chen, Y., Chen, H. and Liu, K. (2012) Response of anaerobic granular sludge to a shock load of zinc oxide nanoparticles during biological wastewater treatment. *Environmental science & technology* 46(11), 5997-6003.

Li, L.-L., Tong, Z.-H., Fang, C.-Y., Chu, J. and Yu, H.-Q. (2015) Response of anaerobic granular sludge to single-wall carbon nanotube exposure. *Water research* 70, 1-8.

Chen, Y., Cheng, J.J. and Creamer, K.S. (2008) Inhibition of anaerobic digestion process: a review. *Bioresource technology* 99(10), 4044-4064.

Angelidaki, I. and Sanders, W. (2004) Assessment of the anaerobic biodegradability of macropollutants. *Reviews in Environmental Science & Bio/Technology* 3(2), 117-129.

Stams, A.J., De Bok, F.A., Plugge, C.M., Eekert, V., Miriam, H., Dolfing, J. and Schraa, G. (2006) Exocellular electron transfer in anaerobic microbial communities. *Environmental microbiology* 8(3), 371-382.

Stams, A.J. and Plugge, C.M. (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nature Reviews Microbiology* 7(8), 568-577.

Lovley, D.R. (2011) Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. *Energy & Environmental Science* 4(12), 4896-4906.

Malvankar, N.S. and Lovley, D.R. (2012) Microbial nanowires: a new paradigm for biological electron transfer and bioelectronics. *ChemSusChem* 5(6), 1039-1046.

Shrestha, P.M., Rotaru, A.E., Aklujkar, M., Liu, F., Shrestha, M., Summers, Z.M., Malvankar, N., Flores, D.C. and Lovley, D.R. (2013) Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy exchange. *Environmental microbiology reports* 5(6), 904-910.

Summers, Z.M., Fogarty, H.E., Leang, C., Franks, A.E., Malvankar, N.S. and Lovley, D.R. (2010) Direct exchange of

electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* 330(6009), 1413-1415.

Nagarajan, H., Embree, M., Rotaru, A.-E., Shrestha, P.M., Feist, A.M., Palsson, B.Ø., Lovley, D.R. and Zengler, K. (2013) Characterization and modelling of interspecies electron transfer mechanisms and microbial community dynamics of a syntrophic association. *Nature communications* 4.

Rotaru, A.-E., Shrestha, P.M., Liu, F., Shrestha, M., Shrestha, D., Embree, M., Zengler, K., Wardman, C., Nevin, K.P. and Lovley, D.R. (2014) A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. *Energy & Environmental Science* 7(1), 408-415.

Liu, F., Rotaru, A.-E., Shrestha, P.M., Malvankar, N.S., Nevin, K.P. and Lovley, D.R. (2012) Promoting direct interspecies electron transfer with activated carbon. *Energy & Environmental Science* 5(10), 8982-8989.

Kato, S., Hashimoto, K. and Watanabe, K. (2012a) Methanogenesis facilitated by electric syntrophy via (semi) conductive iron-oxide minerals. *Environmental microbiology* 14(7), 1646-1654.

Kato, S., Hashimoto, K. and Watanabe, K. (2012b) Microbial interspecies electron transfer via electric currents through

conductive minerals. *Proceedings of the National Academy of Sciences* 109(25), 10042-10046.

Cruz Viggi, C., Rossetti, S., Fazi, S., Paiano, P., Majone, M. and Aulenta, F. (2014) Magnetite particles triggering a faster and more robust syntrophic pathway of methanogenic propionate degradation. *Environmental science & technology* 48(13), 7536-7543.

Alkaya, E. and Demirer, G.N. (2011) Anaerobic-fed and sequencing-batch treatment of sugar-beet processing wastes: a comparative study. *Water Environment Research* 83(3), 247-255.

Speece, R.E. (1996) *Anaerobic biotechnology for industrial wastewaters*.

Sheng, G.-P., Yu, H.-Q. and Li, X.-Y. (2010) Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. *Biotechnology advances* 28(6), 882-894.

Kang, S., Pinault, M., Pfefferle, L.D. and Elimelech, M. (2007) Single-walled carbon nanotubes exhibit strong antimicrobial activity. *Langmuir* 23(17), 8670-8673.

Pacurari, M., Yin, X.J., Zhao, J., Ding, M., Leonard, S.S., Schwegler-Berry, D., Ducatman, B.S., Sbarra, D., Hoover, M.D. and Castranova, V. (2008) Raw Single-Wall Carbon Nanotubes Induce Oxidative Stress and Activate MAPKs, AP-1, NF-[kappa]

B, and Akt in Normal and Malignant Human Mesothelial Cells. Environmental health perspectives 116(9), 1211.

Pasquini, L.M., Sekol, R.C., Taylor, A.D., Pfefferle, L.D. and Zimmerman, J.B. (2013) Realizing comparable oxidative and cytotoxic potential of single-and multiwalled carbon nanotubes through annealing. Environmental science & technology 47(15), 8775-8783.

Tong, Z., Bischoff, M., Nies, L.F., Myer, P., Applegate, B. and Turco, R.F. (2012) Response of soil microorganisms to as-produced and functionalized single-wall carbon nanotubes (SWNTs). Environmental science & technology 46(24), 13471-13479.

Schink, B. (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and molecular biology reviews 61(2), 262-280.

Stams, A.J. (1994) Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek 66(1-3), 271-294.

Ishii, S.i., Kosaka, T., Hori, K., Hotta, Y. and Watanabe, K. (2005) Coaggregation facilitates interspecies hydrogen transfer between *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus*. Applied and Environmental Microbiology 71(12), 7838-7845.

- Kato, S., Nakamura, R., Kai, F., Watanabe, K. and Hashimoto, K. (2010) Respiratory interactions of soil bacteria with (semi) conductive iron- oxide minerals. *Environmental microbiology* 12(12), 3114-3123.
- Jiang, L., Wang, J., Liang, S., Cai, J., Xu, Z., Cen, P., Yang, S. and Li, S. (2011) Enhanced butyric acid tolerance and bioproduction by *Clostridium tyrobutyricum* immobilized in a fibrous bed bioreactor. *Biotechnology and bioengineering* 108(1), 31-40.
- Lynd, L.R., Weimer, P.J., Van Zyl, W.H. and Pretorius, I.S. (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and molecular biology reviews* 66(3), 506-577.
- MallaáShrestha, P. (2014) A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. *Energy & Environmental Science* 7(1), 408-415.

Table 1 Beet sugar industrial wastewater characterized index (all measurements are in mg L⁻¹ except pH)

Component	Value
pH	6.8
SS	498.0 ± 2.1
VSS	186.0 ± 1.2
TOC	840.0 ± 0.8
Cl ⁻¹	97.3 ± 0.2
SO ₄ ²⁻	171.8 ± 1.2
NO ₃ ⁻	92.6 ± 0.7
TP	9.5 ± 1.5
K	144.7 ± 0.5
Ca	306.1 ± 0.4
Na	85.6 ± 0.1
Mg	12.5 ± 1.4
Al	0.4 ± 0.1
Fe	0.8 ± 0.2
COD	3000.0 ± 300.5

Fig. 1 Schematic diagram of three EGSB reactors: (a) control, (b) IONPs, and (c) MWCNTs, each connected to influent tank and configured with ORP detector, temperature detector and controller, influent tank, influent, recycling pumps, effluent tank and gas bag.

Fig. 2 profiles of COD changes in anaerobic degradation of beet sugar industrial wastewater in EGSB reactors for the control, IONPs and MWCNTs

Fig. 3 effects of IONPs and MWCNTs on (a) biogas (b) cumulative biogas and (c) cumulative methane production at experimental time of 74 days

Fig. 4 -Changes of H_2 content in the headspace (a), acetic acid (b) and propionic acid concentrations (c) in the anaerobic digestion of beet sugar industrial wastewater

Fig. 5 phylogenetic representation of archaea and bacteria in control, IONPs and MWCNTs EGSB loaded reactors. Only phylotypes of relative abundance of at least 0.5 % in either of the reactors are shown in: (a) phylum, (b) class and (c) order, while only those with at least 1 % abundance in either of the reactors was shown in (d) genus

Fig. 7 Distribution profiles of EPS in the control (a1-a4), IONPs (b1-b4) and MWCNTs (c1-c4) reactors from the outer surface of the AGS. Sludge granules were simultaneously stained with FITC (proteins-green, [a1, b1, and c1]), Con A (α -D-glucopyranose polysaccharides, red, [a2, b2 and C2]) and calcofluor white (β -D glucopyranose polysaccharides, blue, [a3, b3 and c3]). An overlay of all the stains used as shown in a4, b4 and c4.