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ARTICLE

Efficient degradation of sulfamethoxazole and the responses of microbial community in microbial fuel cells

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Sulfamethoxazole (SMX) is an extensively consumed sulfonamide antimicrobial agent and frequently detected in surface water. This work studied the degradation process of SMX in anodic chamber of microbial fuel cell (MFC) reactors. We found that the biodegradation of SMX could be achieved after acclimation and even high concentration of SMX (e.g. 200 ppm) could be rapidly degraded. Excitation and emission matrix fluorescence spectroscopy analysis revealed that the chemical structure of SMX was decreased during the process, Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer was used to identify the degradation byproducts of SMX. The activity of electrode biofilm was examined afterwards and we found that the microbes were in active state with the antimicrobial agent. High-throughput sequencing suggested that the microbial community structure greatly changed during the operation; some reported SMX scavengers, such as *Achromobacter* and *Pseudomonas,* were abundant in reactors. Some metazoans were also recognized in biofilm samples which indicate that the operation of MFC reactors was in steady state. This study discussed the degradation mechanism of SMX and explored the microbial community response during the process, which provided useful information for the applying of MFC in antibiotics elimination.

Introduction

Accompanied with the wide application of antibiotics, large amount of antibiotics were discharged into sewage system. Since the antibiotics were hard to be biodegraded, it could not be effectively removed through conventional wastewater treatment technology. Sulfamethoxazole (SMX) is one of the extensively used antibiotics and detected in environmental samples[1-3]. Schaider et al. detected SMX in US drinking water supply wells with the concentration up to 113 ppt [4]. The concentration of SMX in surface water ranged from 7.9 to 1900 ppt [5-7] and in groundwater ranged from 38 to 450 ppt [8, 9].

To eliminate the negative effects of SMX, degradation was a favored process. Bio-degradation hold advantages in operation cost, robustness and application scope compared with chemical oxidation methods, but biodegradation was a time consuming process and would be influenced by the concentration of organics. Many researchers had studied the bio-degradation process of SMX. Jiang et al[10] had isolated *Pseudomonas psychrophila* HA-4 which could utilize SMX as sole carbon and energy source, and 34.3% of SMX could be degraded after 8 days operation. Bouju et al[11] isolated five strains of SMX degradation microbes from a membrane bioreactor and found that SMX could be partly mineralized after 16 days operation. Jesús García-Galán et al[12] found that white-rot fungus *Trametes versicolor* could degrade 9 ppm SMX after 20 hours culture and Muller et al[13] reported that SMX could be effectively degraded in activated sludge system. However, there were few reports about the rapid degradation of SMX in sewage treatment reactors and the effects of SMX concentration towards SMX degradation was still ambiguous.

Microbial fuel cell (MFC) technology aroused increasing interest in recent years for its performance in energy recycling and pollutants degradation. Many published researches revealed that MFC could improve the removal rate of pollutants in sewage[14] and soil[15, 16]. In our previous study, we also found that utilizing MFC to degraded SMX was a feasible solution[17]. However, weather the degradation of SMX relay on its original concentration and the long term performance of MFC towards SMX removal still bothered us. Moreover, the microbial community structure change during the process may also offer useful information for further study about the degradation of SMX.

In this study, we initially studied the removal process of SMX and explored the relationship of removal rate with SMX concentration. Then the degradation byproducts were examined based on mass spectrometer and the metabolic pathway of SMX was proposed. To further understand the mechanism, the activity of MFC biofilm was examined by fluorescent staining method and high-throughput sequencing was employed to analyze the microbial community composition. The microbial community shift during different operation stage revealed how microbial community responded to the substrate change. These results afford useful information for further study about SMX degradation microbial strains.

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Materials and methods

2.1 Chemicals

Sulfamethoxazole was ordered from Sigma-Aldrich (Munich, Germany); all other chemicals were purchased from Aladdin Industrial Corporation (Shanghai, China). Ultrapure water was prepared from Milli-Q 18 MΩ system (Millipore, Germany). Water sample was filtered through a 0.22-μm-pore-size filter (Millipore, Germany) before each test. SMX was dissolved in methanol with a concentration of 25 mg/mL and stored as stock solution in dark place.

2.2 Reactors operation

Two-chamber perspex MFC reactors were used in this study. The working volume of each chamber was 115 ml. Anodic and cathodic chamber were separated by a cation exchange membrane (4.0× 4.0 cm^2, Zhejiang Qianqiu Group Co., Ltd., China). Carbon felt (4.0× 4.0 cm^2, Haoshi Carbon Fiber Co., Ltd., China,) was used as electrode and connected with titanium wire (1 mm in diameter, Shanghai GuiTai Titanium Group Co., Ltd., China). Anodic chamber was inoculated with anaerobic sludge and the anolyte was changed in batch mode. Cathodic chamber was filled with potassium ferricyanide (100 mM, pH 7.0) and changed biweekly. The voltage of reactors were monitored by digital multi-meter (Keithley Instruments, Inc., USA). All reactors were set in thermostat container (35 ± 2 °C).

Initially, artificial wastewater containing sodium acetate (NaAc,1g/L), phosphate buffered solution (20 mM, pH=7.0) and trace elements solution[18] was injected into anodic chamber to start MFC reactors. After one month's operation, the voltage output of reactors were maintained at steady level (590 ± 30 mV). Then SMX stock solution (end concentration: 20 ppm) was injected into reactors instead of NaAc, and the anolyte was changed biweekly. Six months later, the added SMX could be removed within a week and the anolyte was changed weekly. Twelve months later, we found that SMX could be rapidly removed in all reactors (within 3 days) and the concentration of SMX was increased from 20 ppm to 50 ppm. Fourteen months later, the concentration of SMX in reactors were increased to 150 ppm and further added into 200 ppm after eighteen months operation.

2.3 Morphology Analysis of Biofilm.

Field emission scanning electron microscopy (S4800, Hitachi, Japan) was used to study the morphology character of electrode biofilm. MFC anode samples were rinsed with 20 mM PBS (pH 7.0) 3 times, fixed by 5 wt.% glutaric aldehyde in 50 mM PBS (pH 7.0), and dehydrated in ethanol aqueous solution (25%, 15min; 50%, 15min; 75%, 15min; 90%, 20min; 95% 20min and 100% 20min). Then the samples dried supercritically and coated with gold for SEM observation.

SYBR®Green I and Propidium Iodide (Invitrogen, Carlsbad, USA) were utilized to perform a double stain for the electrode biofilm. Ten microliters of 30 mM PI was dissolved in DMSO and mixed with 1000 μL of SYBR®Green I. The mixture was diluted 100-times as stock solution. Biofilm samples were tore from MFC anode and rinsed with 20 mM PBS (pH 7.0) three times to remove loose biomass debris and extracellular polymeric substances. Then the sample were stained with 10 μL stock solution and incubated in the dark at room temperature for 20 min, and rinsed again with ultrapure water to remove excess dye. Inverted fluorescence microscope (IX71, Olympus, Japan) was used to detect the samples.

2.4 Three dimensional excitation and emission matrix (3D-EEM) fluorescence spectroscopy analysis

EEM spectra were measured with an F-4600 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). EEM spectra were recorded with scanning emission spectra (from 230 to 600 nm at 10 nm interval) by changing the excitation wavelength (from 200 to 500 nm at 10 nm interval). Excitation and emission slits were set at 5 nm, and the scanning speed was maintained at 240 nm/min. The PMT voltage was set at 700V. The spectrum of 20mM PBS was recorded as blank control.

2.5 Analysis of SMX degradation byproducts

The reactor effluent samples were detected with a Thermo Scientific Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (San Jose, CA, USA) under positive electrospray mode. Chromatography was performed with an ultra-performance liquid chromatograph (UPLC, Waters, USA) system at a flow rate of 200 μL/min on a Waters C18 column (Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm). Acetonitrile was used as mobile phase. The collision energy was set as 28.0 for HCD fragmentation.

2.6 Microbial community analysis

In order to study the microbial community change during the SMX removal process, bio-samples at different operation stage were collected. Stage 1, raw sludge, which was inoculated into reactors. Stage 2, MFC biofilm samples which utilizing NaAc as substrate. The samples were collected from anode after one month's operation. Stage 3, MFC biofilm samples which using SMX as substrate. The samples were collected from anode after 10 month's operation. All samples were rinsed with ultrapure water and stored at -20℃ for further analysis.

Electrode biofilm samples were torn from the top, middle and bottom part of the carbon felt. After that the samples were mixed and minced with sterile scalpel. The DNA extraction and purification were carried as previously reported[19]. Extracted DNA samples were quantified with micro-ultraviolet spectrophotometer (Nanodrop Inc., USA). Purified DNA samples were used as templates for PCR amplification as previously reported[20]. The PCR products were quantitated with QuantiFluor™-ST and mixed for Illumina Miseq sequencing.

Quantitative insights into microbial ecology (QIIME, version 1.8) was used to process the Miseq data of 16S rRNA gene sequences. Miseq data was merged before data analysis and the completeness of barcodes and primer sequencing was checked by QIIME. The reads which were shorter than 200 bp and quality score below 25 was removed. After that the sample sequences were labeled with 6 bp barcodes respectively. Only those sequences with above 97% identity were divided into OTUs for further data analysis. The most abundant sequence of each OTU was defined as the representative sequence and assigned by PyNAST, and these representative sequences were employed for the classification of taxonomic according to Greengenes database. We randomly chosen 13056 sequences per sample to explore the α-diversity, and compared β-diversity between samples. The account of OTUs, Chao1 index, Shannon index and phylogenetic diversity index of each sample was calculated.

Results and discussion

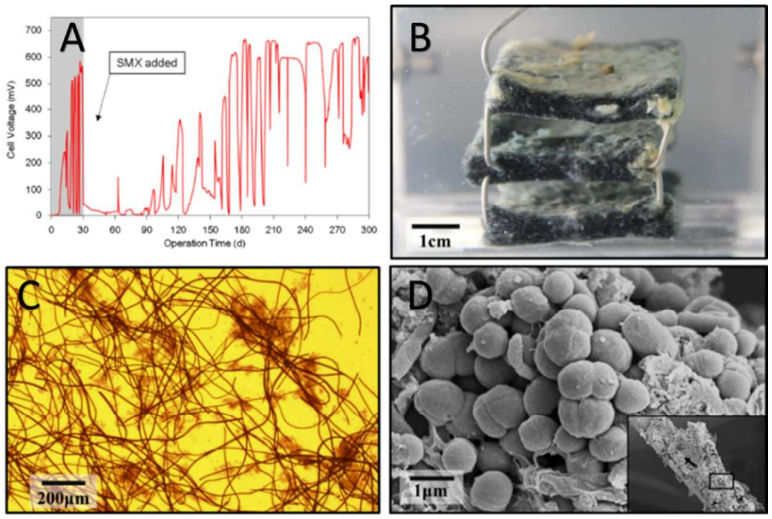

Figure 1. The voltage fluctuation of an MFC reactor after inoculation as a function of time (A), photo of MFC biofilm-electrode (B), microscope photograph of electrode and biofilm (C) and scanning electron microscopic image of biofilm-electrode (D).

3.1 Reactor startup and operation

Since we focused on the biodegradation of recalcitrant contaminants, the function of microbe is vital. Anaerobic sludge was collected from a coal gasification wastewater treatment plant[21] which mainly disposes phenolics, poly-nuclear aromatic hydrocarbons and heterocyclic compounds. The anaerobic sludge was inoculated into MFC reactors and NaAc was fed as substrate. Figure 1A illustrated a reactor's voltage fluctuation curve during the operation process. Reactor voltages were soared to 260 ± 50 mV after several day's operation. Considering the voltage of MFC was related with the activity of electrochemically active biofilm, the rapid increasing of cell voltage revealed the success startup of MFC system. After several operation cycles, the effluent was turbid and bubbles were formed on anode. During this period, the output voltages were maintained at 580 ± 30 mV, indicating that electrochemically active biofilm was forming and the microbes had vigorous metabolism.

After a month's operation, the output voltage of reactors were in a steady state. Then NaAc was replaced with SMX stock solution (20 ppm SMX). We found that the turbidity of anolyte was greatly reduced and the effluent became odorless, with the voltages dropped to 15 ± 6 mV. As a broad-spectrum antibacterial agent, it seems that SMX had greatly inhibited the activity of microorganism. However, during the 2-4 months of the operation process, the voltage of reactors was gradually increasing. Five months later, the voltages stably raised to 640 ± 40 mV at each batch operation, indicating that the microbes in MFCs may utilize SMX for metabolism and electricity production.

The surface morphology of anode after 10 months of operation is shown in Figure 1B. Biofilm was uniformly attached on the carbon felts and bubbles could occasionally form on the biofilm. We tore some biofilm from the carbon felt electrode and examined it with microscope (Figure 1C). It was found that the biofilm was firmly attached on the carbon felt fibers. Besides, the strong connection between biofilm and electrode was in favor for the energy production of MFC system. Figure 1D showed a microscopic morphology of the electrode biofilm. Different genus of microorganism (Sarcina, Diplococcus, Coccus, Bacillus etc., mixing with cell debris and secretion) could be observed in this view, suggesting that the community structure of the biofilm was rich.

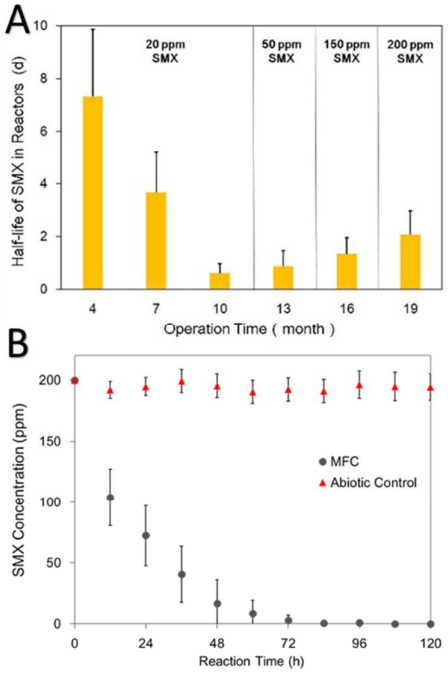

Figure 2. Half-life time of SMX in reactors as a function of time (A) and concentration change of 200 ppm SMX in MFCs and abiotic control after 18 months' operation (B).

The concentration of SMX in supernatant was monitored during the whole experiment. After months of operation, the removal ability towards SMX was gradually increasing. After data analyzing and fitting, and compared with studies[22, 23] about the biodegradation of recalcitrant chemicals, we infer that the concentration change of SMX was following (pseudo) first order dynamics. Figure 2A showed the half-life time of SMX in MFC reactors as a function of time. Four months later, 50% SMX (20 ppm) could be removed in MFC reactors within 10 days. Ten months later, the half-life of SMX

in reactors shorted to 1 day, and 20 ppm SMX could be fully removed within 3 days. These data suggested that the high removal efficiency of SMX could be enhanced with acclimation.

Since the added SMX (20 ppm) were rapidly removed in reactors, whether the reactors could remove higher concentration of SMX aroused our interest. The concentration was gradually increased to 200 ppm after 18 months' operation. Around 70% SMX could be removed within 24 hours and its concentration was below detection limit after 96 hours as shown in Figure 2B. However, the concentration of SMX showed no obvious change in abiotic control reactors. When the concentration increased from 20 ppm to 200 ppm, the half-life time of SMX in reactors merely increased from 1 day to 3 days. Xu et al. also noticed that the degradation of SMX in their bioreactors was not dependent on its original concentration, and speculated that co-metabolism may happen in the biodegradation of SMX[24].

3.2 Degradation mechanism of SMX

In above section we discussed the concentration change of SMX in reactors. However, whether the SMX had been degraded was still uncertain. Fluorescence EEM analysis was carried to study the structure change of SMX. Figure 3 illustrated the fluorescence characteristic of reactor supernate sampled at different time. Peak A

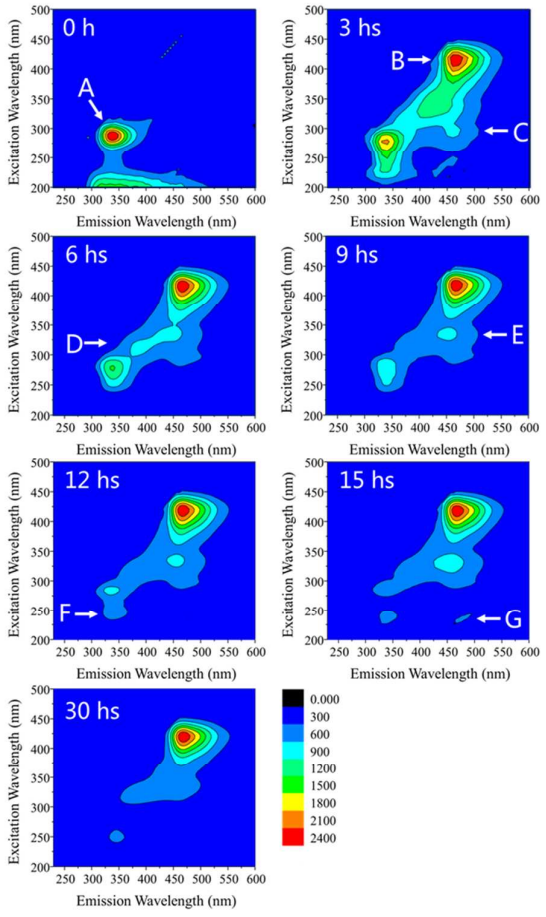

Figure 3. EEM fluorescence spectra of MFC anolyte sampled at different time (subtracted blank to eliminate scatter); peak A ($\lambda_{ex}/\lambda_{em}$ =290/340) refers to SMX; peak B ($\lambda_{ex}/\lambda_{em}$ =420/470) refers to coenzyme F420; peaks C, D, E, F refer to degradation products. The intensity of peak A was decreased among samples, which reveals the elimination of SMX in MFC.

($\lambda_{ex}/\lambda_{em}$=290/340) could be attributed to SMX since the influent contained no other organics and there was no fluorescence peak in blank sample (i.e. reactor influent without SMX). The intensity of peak A was decreased and completely vanished after 30 hours operation, while the intensity of peak B ($\lambda_{ex}/\lambda_{em}$=420/470) was rising. This phenomenon revealed that the chemical structure of SMX and metabolism byproducts were changing as a function of time, and it could be attributed to the degradation behavior. A few scattered peaks (C, D, E and G) were formed and then vanished during the operation process, indicating the transformation of fluorescence exciting chemicals. Previous studies[25, 26] reported that peak B belongs to coenzyme F420, a kind of coenzymes act as an electron carrier in both anabolic and catabolic redox reactions[27] of methanogenic bacteria. Considering the fact that the operation of MFCs is generally accompanied with the formation of electron transfer mediators, we speculate that some degradation products of SMX might be utilized by microbe to synthesis exoenzyme or other electrochemical active substances. Moreover, coenzyme F420 may enhance the bioelectrochemical process of our systems as a kind of electron transfer mediator.

To further understand the degradation pathway of SMX, Orbitrap mass spectrometer was employed to detect the degradation byproducts. Since the detection was carried under positive mode, organics were combined with cation and the detected m/z values were 1 unit (-NH_2 combined with H^+), 22 units (-COOH combined with Na^+) or 38 units (-COOH combined with K^+) higher than the original molecule. Several potential metabolites were identified from the total ion current chromatogram spectra (Figure 4A). Previous studies reported that 3-amino-5-methylisoxazole (3A5MI) appeared during the degradation of SMX, and we also found 3A5MI in the samples (peak c, m/z 99.0528). The m/z difference of peak 4 and peak 5 was 22.0001, indicating that chemical 5 may be the sodium

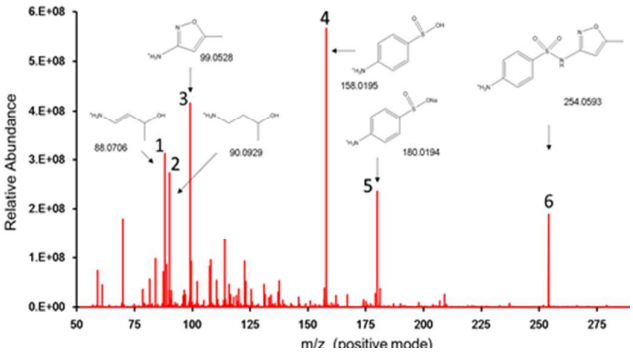

Figure 4. Q-Exactive spectrum of MFC effluent sample

salt of chemical 4, and chemical 4 was a kind of organic acid. We speculated that chemical 4 was 4-Amino benzene sulfinic acid. During the degradation process, we noticed that 3A5MI could be further degraded in reactors, and speculated that chemical 1 was a degradation product of 3A5MI. Based on the identified degradation byproducts, a degradation pathway of SMX in MFC reactor was proposed. SMX was initially hydrolyzed into 4-Amino benzene sulfinic acid and 3A5MI. Then the isoxazole ring of 3A5MI was opened and its nitrogen atom was removed, result in the formation of 4-amino-2-butanol. Since the 4-amino-2-butanol is a chain structure, it is easy for microbial degradation.

3.3 Microbial analysis

With the acclimation, the electrode biofilm was getting more and more thick, it may influence the activity of microbes and hinder the mass transfer from solution into biofilm (bred in the middle part of carbon felt electrode). The activity of biofilm was estimated by fluorescent staining method (Figure 5). Most of the cell presented intense green fluorescence, indicating that their morphology structure was intact and they might be in active state. Orange spots represent damaged cells which might be dead or in apoptotic state, and there was few microbes in this state. Moreover, a layer of active microbe uniformly attached on the surface of carbon felt fiber (with some floc twisted around it). This phenomenon indicates that microbes bred in MFC carbon felt posed a great activity.

Interestingly, during the microscopic examination of biofilm samples, we noticed that some suspected metazoans such as roundworm (Figure 5A and Figure S1) may exist in reactors. Generally, the appearance of metazoans reveals that food chains had formed in bioreactors and the water quality was not bad[28]. Besides, metazoans could devour cell debris and facilitate the regeneration of biofilm. It seems that the degradation products in MFCs may not further hinder the growth and breed of metazoans.

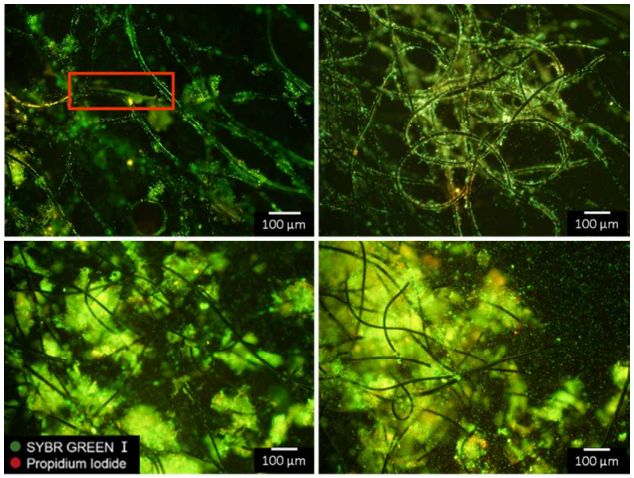

Figure 5. Microscopic fluorescence photo of MFC biofilm samples. Green fluorescent may emitted from intact cell while red fluorescent may emitted from damaged cell. (Red frame instruct suspected roundworm)

According to the High-throughput sequencing results in Figure 6, the OTU number of raw sludge was the largest compared with other samples, indicating that the microbial composition of raw sludge was rather complex. Since the raw sludge was disposing coal gasification wastewater, diverse microbial species was in favor for the degradation of complex substrates. However, when the substrate was changed to NaAc or SMX while the reactor was operated under MFC mode, the diversity of microbial community was largely decreased. This may be partly ascribed to the selectivity of MFC system, and the substrate was simple for microbe degradation and hence influenced the community structure. In addition, since the bio-sample in NaAc stage and SMX stage was biofilm, while the sample of stage 1 was sludge, the difference in operation mode could also greatly influence the community structure. According to the

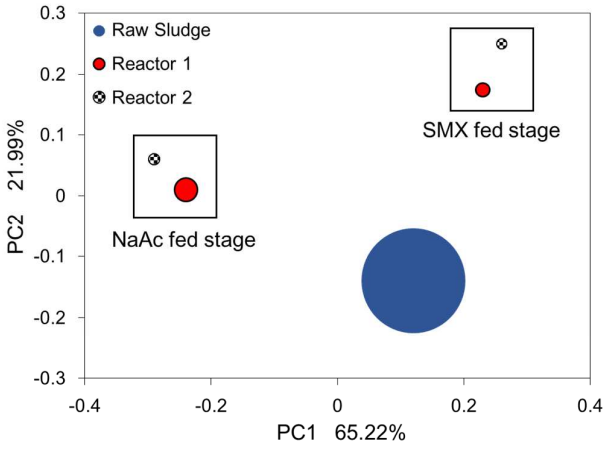

Figure 6. Weighted principle coordinate analysis (PCoA) of microbial communities sampled at different stage. The size of the dot represents the OTU number of each sample.

PCoA result, we noticed that for different reactors, when they were fed with same substrate (NaAc or SMX), the bio-samples tended to cluster together. This indicating that for the consumption of different substrates, certain species may hold advantage and they may gradually dominant in the microbial community. On the other hand, the distance between the samples which fed with SMX or NaAc was rather great, revealing that substrate had great influence on the composition of microbial community.

Bio-samples of raw sludge, NaAc as substrate and SMX as substrate were examined by high-throughput sequencing method to study the shifts in microbial community structure. Figure 7A showed the microbial phyla of bio-samples collected at different stage. In raw sludge, Proteobacteria (49%) was the largest phylum, suggesting that Proteobacteria may contribute a lot in the disposal of coal gasification wastewater. However, when the substrate was changed and operated under closed circuit mode, the community share of Proteobacteria was decreased to 25.4~33.6%. On the other hand, Euryarchaeota (1.7%) was the smallest phylum in raw sludge. But it was increased to 16.2~21.7% in MFC when the reactor was fed with SMX. The relative abundance of Planctomycetes in raw sludge account for merely 8%. When the NaAc was fed into MFC reactors, the relative abundance of Planctomycetes was increased to 25% around. And its abundance decreased to 7% in biofilm when SMX was fed as substrate.

The heat map (background image) of Figure 7B present the relative abundance of microbial phylum while the size of bubbles in chart represent the relative abundance of different microbial genera identified in the samples. Generally, the relative abundance of different microbial genera in raw sludge was balanced, but the relative abundance of some genera was largely increased when fed with NaAc (*Mathanosarcina* and *Mycobacterium*) or SMX (*Methanobacterium*, *Methanosaeta* and *Treponema*). This may be that the carbon source of raw sludge was rather complex, and different genus were fertilized together to maintain a balance towards substrate degradation.

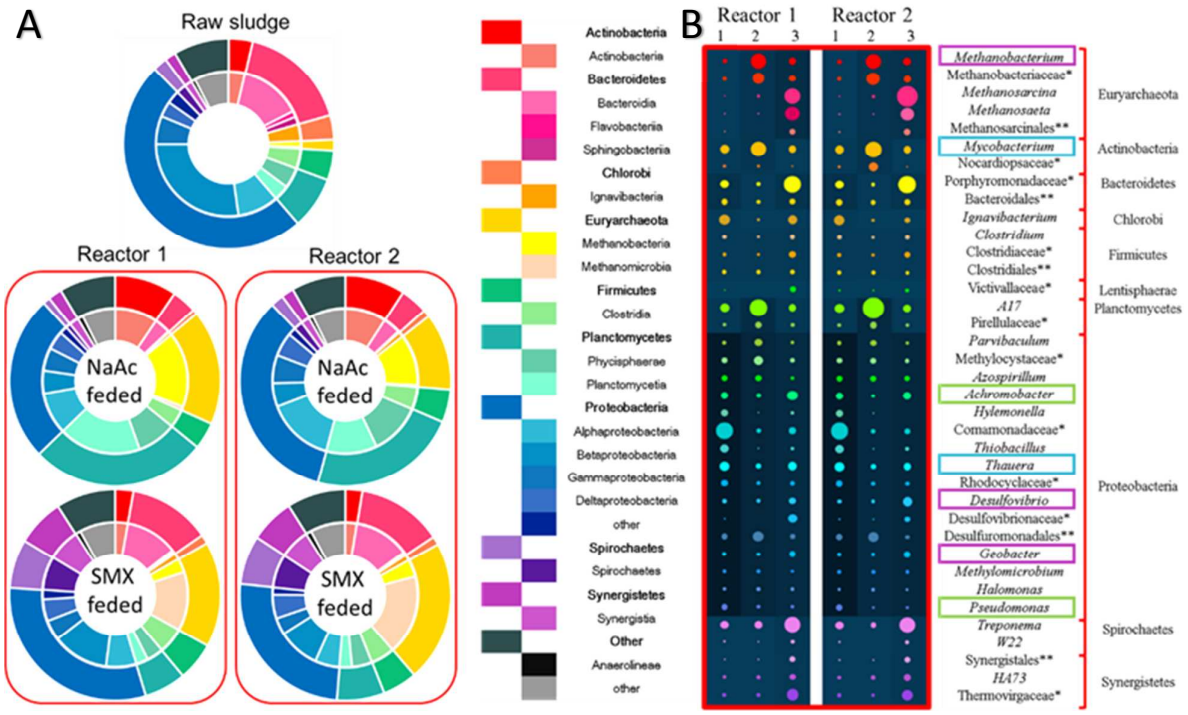

Figure 7. Taxonomic classification according to microbes' DNA sequences from bio-samples of raw sludge and MFC biofilm at phylum level (A), the outer circles represent the microbe on phylum level (relative abundance > 3%) while the inner circles represent the microbe on class level (relative abundance > 1%); Bubble chart of microbial DNA sequence on genus level (relative abundance > 0.5%) (B); green boxes indicate SMX degradation related microbes; blue boxes indicate phenolic compounds degradation related microbes; violet boxes indicate electrochemical active microbes; Untitled microbes were identified by their family name (*) or order name (**); the numbers on each column of the bubble chart represent the kind of bio-sample:1, raw sludge; 2, NaAc fed biofilm; 3, SMX fed biofilm.

Standing on genus level, *Methanosarcina*, *Mycobacterium* and other two unnamed genera (respectively belong to Methanobacteriaceae and Pirellulaceae) were largely bred in MFCs when fed with NaAc. Theoretically, methane-producing adversely affected energy production in MFCs, since the reduction of organics into methane require electrons. But it would benefit pollutants degradation, different consortia had to compete for substrate utilization and this may lead to more effective degradation performance towards recalcitrant chemicals. Besides, the formation of methane-producing consortia also revealed that macromolecules could be degraded into small organics in the system. When the substrate was changed from NaAc to SMX, microbial community changed greatly. The copy number of *Methanobacterium*, *Methanosaeta*, *Treponema*, *Achromobacter* and an unnamed genus (belong to Porphyromonadaceae family) was largely increased. As the SMX is an effective antimicrobial agent, the appearance of methanogens reflects that the biotoxicity of SMX could be effectively removed.

Methanobacterium and *Methanosaeta* may also contribute a lot to mineralization about some degradation products of SMX; the formation of coenzyme F420 based on 3D-EEM test may also relate with their metabolism activity. Some species of *Achromobacter* and *Pseudomonas* hold a strong degradation ability towards SMX according to published results[10, 29] and their appearance in reactors connected with our previous discussion that SMX could be biodegraded in MFCs. Besides, *Mycobacterium*, *Ignavibacterium*, and *Thauera* also had higher relative abundance in the biofilm samples. These genera were generally found in phenol-contaminated soil and sewage systems and capable for degradation of aromatic hydrocarbons[30-32]. The flourish of these microbes may reveal that functional groups serving for SMX degradation had formed in the reactor biofilm and this may relate with the rapid degradation performance of MFC towards SMX.

Conclusions

In this study, we explored the possibility by utilizing MFC reactors to remove SMX and studied the chemical and biological mechanism during the process. We found that SMX could be biodegraded in MFCs after acclimation and even high concentration of SMX (i.e. 200 ppm) could be removed. The degradation process was studied by 3D-EEM and Orbitrap mass spectrometer, with the speculation of degradation mechanism subsequently. After that we examined the activity of MFC biofilm and noticed that some suspected metazoans were existed in the reactors, revealing that the removal performance of MFC was great. High-throughput sequencing technology was used to study the microbial community shift during the SMX elimination process and some reported SMX degradation microbe was identified in the system. This study offers a feasible choice for the elimination of SMX in wastewater and may extended to other antibiotics.

Acknowledgements

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