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1557 Spatial and vertical variations of perfluoroalkyl substances in sediments of the Haihe River, China
Xiuli Zhao, Xinghui Xia, Shangwei Zhang, Qiong Wu, and Xuejun Wang

1567 Dissolved organic matter removal using magnetic anion exchange resin treatment on biological effluent of textile dyeing wastewater
Jun Fan, Haibo Li, Chendong Shuang, Wentao Li, and Aimin Li

1575 Reduction and characterization of bioaerosols in a wastewater treatment station via ventilation
Xuesong Guo, Panpan Wu, Wenjie Ding, Weiyi Zhang, and Lin Li

1585 Distribution and spectral characteristics of chromophoric dissolved organic matter in a coastal bay in northern China
Guiju Li, Jing Liu, Yulan Ma, Ruihua Zhao, Suzheng Hu, Yijie Li, Hao Wei, and Huixiang Xie

1597 Application of fish index of biological integrity (FIBI) in the Sammenxia Wetland with water quality implications
Hong Zhang, Baoqing Shan, and Liang Ao

1605 Analysis of the bacterial community composition in acidic well water used for drinking in Guinea-Blissau, West Africa
Ana Machado, and Adriano A. Bordalo

1615 Performance and role of N-acyl-homoserine lactone (AHL)-based quorum sensing (QS) in aerobic granules
Yaihchen Li, Junping Lv, Chen Zhong, Wen Hao, Yaqin Wang, and Jianrong Zhu

1623 Adsorption behavior of sulfamethazine in an activated sludge process treating swine wastewater
Weiwai Ben, Zhiran Qiang, Xiaowei Yin, Juhe Li, and Xun Pan

1631 Scenario analysis of energy-based low-carbon development in China
Yun Zhou, Fanghua Hao, Wei Meng, and Jiefeng Fu

1641 Chemical characteristics of size-resolved aerosols in winter in Beijing
Kang Sun, Yu Gu, Qiong Wu, Tingting Han, Jianwei Gu, Jingjing Zhao, Yile Sun, Qi Jiang, Zhiyao Gao, Min Hu, Yuanzhang Zhang, Keding Lu, Stephan Nordmann Yafang Cheng, Li Hou, Hui Ge, Masami Funuchi, Mitsuhiko Hata, and Xingang Liu

1651 On-board measurement of emissions from liquefied petroleum gas, gasoline and diesel powered passenger cars in Algeria
Saidene Chikhi, Mounir Bougheddaoui, Rabah Kerbachi, and Robert Jourard

1661 Evaluation of soil washing process with carboxymethyl-ß-cyclodextrin and carboxymethyl chitosan for recovery of PAHs/heavy metals/fluorine from metallurgical plant site
Mao Ye, Mingming Sun, Fredrick Oroni Kengara, Jingting Wang, Ni Ni, Li Wang, Yang Song, Xinglun Yang, Huiqin Li, Feng Hu, and Xin Jiang

1673 Application of sewage sludge and intermittent aeration strategy to the bioremediation of DDT- and HCH-contaminated soil
Qi Liang, Mei Lei, Tongbin Chen, Jun Yang, Xiaoming Wan, and Sucai Yang

1681 Acute and chronic toxic effects of Pb2+ on polychaete Perinereis cibuburensis: Morphological changes and responses of the antioxidant system
Yulu Tian, Hongjun Liu, Qixiang Wang, Jian Zhou, and Xueyi Tang

1689 Effect of temperature switchover on the degradation of antibiotic chloramphenicol by biocathode bioelectrochemical system
Deying Kang, Bin Liang, Du-Jong Lee, Aijie Wang and Nanqi Ren
Aims and scope

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CONTENTS

1699 Submerged vegetation removal promotes shift of dominant phytoplankton functional groups in a eutrophic lake
Jing Dong, Kai Yang, Shuangshuang Li, Genbao Li, and Lirong Song

1709 Universally improving effect of mixed electron donors on the CO2 fixing efficiency of non-photosynthetic microbial communities from marine environments
Jiajun Hu, Lei Wang, Shilong Zhang, Yuanqiang Wang, Fengming Jin, Xiaohua Fu, and Huirong Li

1717 Community structure and elevational diversity patterns of soil Acidobacteria
Yuguang Zhang, Jing Cong, Hui Lu, Guangliang Li, Yuanyuan Qi, Xijiang Su, Jizhong Zhou, and Qidong Li

1725 Extraction and characterization of bound extracellular polymeric substances from cultured pure cyanobacteria (Microcystis wesenbergii)
Lizhen Liu, Boqiang Qin, Yuntian Zhang, Guangwei Zhu, Guang Qiao, Li Huang, and Xin Yao

1733 Electrochemical oxidation of 1H,1H,2H,2H-perfluorocarbon sulfuric acid (6:2 FTS) on DSA electrode. Operating parameters and mechanism
Qiongfang Zuo, Xian Li, Feng Yan, Bo Yang, Shubao Deng, Jun Huang, and Gang Yu

1741 Catalytic wet air oxidation of phenol with functionalized carbon materials as catalysts: Reaction mechanism and pathway
Jianbing Wang, Wansuo Fu, Xuwen He, Shaohua Yang, and Wenpeng Zuo

1751 Graphene-supported nanoscale zero-valent iron: Removal of phosphorus from aqueous solution and mechanistic study
Fenglin Liu, Jinghe Yang, Jane Zhuo, Ding Ma, Lili Gan, Bangmi Xie, Pei Wang, and Bo Yang

1763 Characterization of extracellular polymeric substances in the biofilms of typical bacteria by the sulfur K-edge XANES spectroscopy
Huirong Lin, Chongye Kang, Shenghua Zhang, Lei Zheng, Yidong Zhao, and Xin Yu

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Effect of temperature switchover on the degradation of antibiotic chloramphenicol by biocathode bioelectrochemical system

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ABSTRACT

Exposure to chloramphenicol (CAP), a chlorinated nitroaromatic antibiotic, can induce CAP-resistant bacteria/genes in diverse environments. A biocathode bioelectrochemical system (BES) was applied to reduce CAP under switched operational temperatures. When switching from 25 to 10°C, the CAP reduction rate (kCAP) and the maximum amount of the dechlorinated reduced amine product (AMCl, with no antibacterial activity) by the biocathode communities were both markedly decreased. The acetate and ethanol yield from cathodophilic microbial glucose fermentation (with release of electrons) was also reduced. Formation of the product AMCl was enhanced by the biocathode dechloridation reaction compared with that produced from pure electrochemical or microbial dechloridation processes. The electrochemical and morphological analyses of cathode biofilms demonstrated that some cathodophilic microbes could adapt to low temperature and play a key role in CAP degradation. The resilient biocathode BES has a potential for the treatment of CAP-containing wastewater in temperature fluctuating environments.

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Introduction

Antibiotics are emerging environmental micro-pollutants that cannot be completely removed by conventional wastewater treatment processes. Sewage treatment plants are important point sources of antibiotic entry into the environment (Leung et al., 2012; Zhang and Li, 2011). Exposure to antibiotic substances can result in evolution of multidrug-resistant bacteria and novel antibiotic-resistant genes, which has tremendous ramifications for human health and has attracted global attention (Andam et al., 2011; Davies and Davies, 2010; Le-Minh et al., 2010; Zhang et al., 2009).

The chlorinated nitroaromatic antibiotic chloramphenicol (CAP) is an effective broad spectrum antibiotic that has been widely used since 1950’s. Owing to its fatal toxic effects in humans (e.g., aplastic anemia, potential carcinogenicity and genotoxicity) (Feder et al., 1981; Martelli et al., 1991), CAP has been banned by many countries for use in food-producing animals. However, CAP is still widely used in low-income countries as it is inexpensive and readily available. Trace levels of CAP (ng to μg level) and novel CAP-resistant bacteria/genomes are frequently detected in diverse ecosystems (Leung et al., 2012; Li et al., 2013; Liang et al., 2013; Parsley et al., 2010; Xi et al., 2009; Zhang and Li, 2011; Zhang et al., 2009). Thus, a CAP...
degradation system with high efficiency is needed to decrease the possibility of evolution of CAP-resistant bacteria/genes in the environment.

The bioelectrochemical system (BES) has been developed for the enhanced removal of recalcitrant pollutants. The BES has been applied for cathodic reduction of diverse pollutants (Huang et al., 2011; Rosenbaum et al., 2011), particularly for the reduction of nitroaromatic pollutants (Li et al., 2010; Liang et al., 2013, 2014; Mu et al., 2009; Shen et al., 2012; Sun et al., 2012; Wang et al., 2011, 2012). Bioelectrochemical degradation of CAP by biocathodes and abiotic cathodes was recently studied (Liang et al., 2013; Sun et al., 2013). In general, environmental temperatures vary daily, especially in winter, or at high latitudes and in hilly regions. However, the effects of temperature shift, e.g., dropping 15°C, on the efficiency of bioelectrochemical CAP degradation have not yet been studied. Low temperature would lower rates of conventional anaerobic biological conversion processes (Lettinga et al., 2001). However, the BES could efficiently produce current (Liu et al., 2012a; Michie et al., 2011) and hydrogen (Lu et al., 2011, 2012) at low temperatures (4–15°C). Thus, incorporation of an abiotic cathode in the conventional anaerobic biological reduction process may be a promising process for handling CAP-containing wastewaters with fluctuating ambient temperatures.

This study investigated the chemical kinetics of CAP degradation and product formation subject to an operational temperature switch from 25 to 10°C, with abiotic cathode and open-circuit biocathode BES tests as controls. The cathodophilic microbial role in the CAP transformation was revealed by cyclic voltammetry (CV), confocal laser scanning microscope (CLSM) analysis. The results have proved the effectiveness of reductive degradation of CAP by the biocathode BES with changing operational temperatures.

1. Materials and methods

1.1. BES reactor setup

The BES reactors were constructed from two equal-volume Lexan cubic chambers (4 × 4 × 3 cm) with a cylindrical cavity (3 cm in diameter and 4 cm in length). The internal volume for each chamber was 28 mL and they were separated with a pretreated cation exchange membrane (Ultrex CMI-7000, Membranes International, Ringwood, NJ, USA). Electrodes were made of graphite fiber brush (2.5 cm in diameter and 2.5 cm in length, TOHO TENAX Co., Ltd., Tokyo, Japan). A high-precision resistor (10 Ω) with external power (0.5 V) in series was employed for the connection. The saturated calomel electrode (SCE) model-217, Shanghai Precise Sci. Instrument Co., Ltd., Shanghai, China) was employed as the reference electrode to measure the cathode potential. All of the potential data reported herein are against SHE.

1.2. Microbial inoculum and BES tests

Enrichment of the CAP-reducing consortium was carried out according to Liang et al. (2013). Activated sludge obtained from Taiping Wastewater Treatment Plant (Harbin, China) was used as inoculum. The CAP-reducing microbial consortium was the seed for cathode biofilm development in the BES at 25°C (Liang et al., 2013). Six BES reactors with mature cathode biofilms were operated for a total of six batch-fed cycles (Cooling Incubator, BI-250A, STIK, Shanghai, China) to collect data for the biocathode mode at 25°C (referred to as the RT-biocathode). Subsequently, three reactors were switched to low temperature (10°C) (LT-biocathode in short) (Cooling Incubator, BI-250A, STIK, Shanghai, China) for another six cycles to collect operational data at 10°C. The remaining three reactors were kept at 25°C for another six cycles (a total of 12 cycles for 25°C mode). In biocathode mode, CAP (30 mg/L), glucose (600 mg/L) and the cathode electrode were the cathode electron acceptor, the intracellular electron donor and extracellular electron donor for cathodophilic microbes, respectively. When the electrical circuit of the biocathode BES was disconnected, the open-circuit biocathode test was performed (with only microbial activities with glucose as intracellular electron donor). To collect abiotic CAP degradation data, two other BES reactors with abiotic cathodes were tested for three cycles at 25 and 10°C, respectively. Medium compositions and preparation process for the catholyte and anolyte were as previously described (Liang et al., 2013; Wang et al., 2013).

1.3. Chemicals and analytical methods

CAP (>98% purity) and high performance liquid chromatography (HPLC) grade methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals used in this study were of analytical grade.

Effluent samples taken from the cathode chamber (at 1.5, 6, 12 and 24 hr) were filtered through a 0.45 μm filter before chemical analysis. The concentrations of CAP and the products of CAP transformation were measured using a reverse-phase HPLC (model-2695, Waters, Milford, MA, USA) equipped with a C18 column (5 μm 4.6 × 250 mm, Waters Co., Milford, MA, USA) with UV detection at 250 nm for AMC22 and AMC1, 275 nm for CAP and CAP-acetyl, and 310 nm for the NO intermediate, with UV detection at 250 nm for AMCl2 and AMCl, 275 nm for CAP and CAP-acetyl, and 310 nm for the NO intermediate, respectively. The mobile phase was methanol/H2O (55:45; V/V) at a flow rate of 0.8 mL/min at 30°C (Liang et al., 2013). To identify
the products during CAP transformation, reversed phase liquid chromatography (model-1200, Agilent, Santa Clara, CA, USA) with quadrupole time-of-flight mass spectrometry (QTOFMS) (model-6520, Agilent, Santa Clara, CA, USA) detection in the negative ion mode was carried out on an Agilent Zorbax XDB C18 column (Agilent, Santa Clara, CA, USA) (1.8 μm; 2.1 × 100 mm) with methanol/H2O (55:45; V/V) as the mobile phase at a flow rate of 0.15 mL/min and UV detection at 250 nm (Liang et al., 2013).

Glucose in the biocathode effluent (at 6 hr) was determined by the phenol-sulfuric acid method (Herbert et al., 1971). Volatile fatty acids (VFAs) and ethanol in the biocathode effluent were analyzed using a gas chromatograph (Agilent, 4890D; J&W Scientific, Folsom, CA, USA) with a flame ionization detector (FID) and an appropriate column (19095N-123 HP-INNOWAX, 30 m × 0.53 mm × 1.00 μm, J&W Scientific, Folsom, CA, USA) using nitrogen as carrier gas (Liu et al., 2012b).

The electric current (I) and CAP reduction efficiency (ErCAP, %) were calculated based on the difference between influent and effluent CAP concentrations (Wang et al., 2011). The kinetics of CAP transformation was assumed to follow the first-order reaction model 

\[ C = C_0 \times e^{-kt} \]

where, \( C \) (mg/L) represents the CAP concentration at time \( t \) (hr), and \( C_0 \) (mg/L) is the initial CAP concentration (30 mg/L) (Liang et al., 2013). The rate constant \( k \) (hr⁻¹) calculation and statistical analysis (two-tailed unpaired t-test) were performed by SigmaPlot v.11.0 software (Systat Software Inc., Chicago, IL, USA).

### 1.4. CV and EIS analyses

The CV tests were performed using an electrochemical workstation (WMCP1000K8 Multichannel Potentiostat, WonATech Co., Ltd., Seoul, South Korea) equipped with a three-electrode system. The cathode/biocathode and the anode of the BES were used as the working and counter electrode, respectively. The SCE was the reference electrode. Cyclic voltammograms were recorded with the scan rate of 5 mV/sec.

The EIS tests were carried out over a frequency range of 0.01 to 10⁵ Hz (Kong et al., 2013) and amplitude of 5 mV (WMCP1000K8 Multichannel Potentiostat, WonATech Co., Ltd., Seoul, South Korea) for the analysis of the polarization internal
resistance of the abiotic cathode and biocathode BES under different operational modes. Concentrations of CAP and glucose were 30 and 600 mg/L, respectively, when used in the CV and EIS analyses at 25°C.

1.5. Microscopy analysis

The immobilization and morphology observation of RT-biocathode and LT-biocathode biofilms (both had two biological replicates) were carried out by scanning electron microscopy (SEM) (HELIOS Nanolab 600i, Hillsboro, OR, USA) at 20 kV acceleration voltage according to Xie et al. (2011).

RT-biocathode and LT-biocathode biofilms (both had two biological replicates) were stained with the LIVE/DEAD BacLight Bacterial Viability Kit (L7012) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and then examined using CLSM (CARL ZEISS LSM 700, Oberkochen, Germany).

2. Results and discussion

2.1. Bioelectrochemical degradation of CAP

The CAP reduction rates \( k_{\text{CAP}} \) under four different operational modes were compared. In stage I (biocathode; 25°C), the \( k_{\text{CAP}} \) for all BES biocathodes was close \((p = 0.750, n = 18)\), confirming the consistency of the performance of the six biocathode reactors. When switching the operational temperature of the three test reactors from 25 to 10°C (stage II, LT-biocathode, 10°C vs. RT-biocathode, 25°C), the \( k_{\text{CAP}} \) of the LT-biocathode was markedly decreased \((p < 0.001, n = 18)\) (Fig. 1), suggesting strong temperature effects on CAP reduction activity by the cathodophilic microbes. However, within 24 hr of testing, the Er\( k_{\text{CAP}} \) of the LT-biocathode was close to that of the RT-biocathode \((98.94\% \pm 0.66\%)\). When the circuit of the biocathode was disconnected (stage III, open-circuit biocathode with only microbial activity), the open-circuit RT-biocathode had higher \( k_{\text{CAP}} \) and Er\( k_{\text{CAP}} \) \((p = 0.044, n = 3)\) than those of the open-circuit LT-biocathode \((69.90\% \pm 10.25\%)\) (Fig. 1). Additionally, decrease in operational temperature had no significant effects on \( k_{\text{CAP}} \) or Er\( k_{\text{CAP}} >95\% \) of the abiotic cathode BES \((p = 0.319\) or \(p = 0.195, n = 3\)). This occurrence suggested that anodophilic microbes enriched at 25°C could efficiently generate electrons in the anode and together with the applied external voltage of 0.5 V for CAP degradation at 10°C. The \( k_{\text{CAP}} \) of the open-circuit LT-biocathode was lower than that of the LT-biocathode and of the abiotic cathode \( (p = 0.032\) ± 0.069) had larger \( k_{\text{CAP}} \) than that of the abiotic cathode \((10°C)\) \((0.422\) ± 0.066) \((p < 0.001)\) (Fig. 1). The cathodophilic microbes present could adapt to low temperature stress for CAP degradation.

Fig. 3 – Dynamic characterization of four major CAP degradation product formation (AMCl2, AMCl, NO and CAP-acetyl) under different operational modes.
2.2. Products of CAP degradation

The identification of CAP degradation products (AMC12: reduced amine product; AMC1: dechlorinated AMC1; NO: nitroso intermediate; CAP-acetyl: acetylation of 1-hydroxyl or 3-hydroxyl of CAP) was carried out with LC-QTOFMS and HPLC as demonstrated below. The HPLC peaks for CAP and the corresponding products are shown in Fig. 2a consistent with previous results (Liang et al., 2013). The characteristic two-chlorine-atom isotope clusters for CAP were observed with $\text{m/z}$ of 321, 323 and 325 [$\text{M} - \text{H}$] (the $\text{m/z}$ of 325 showed very low abundance) (Fig. 2b). The dechlorinated amine product AMC1 and amine product AMC12 were identified according to the theoretical $\text{m/z}$ (257 and 259 [$\text{M} - \text{H}$] one-chlorine-atom isotope clusters) and (291, 293 and 295 [$\text{M} - \text{H}$] two-chlorine-atom isotope clusters) (Fig. 2c). The curves for the four main products revealed similar trends in stages I and IV (Fig. 3a–d). The maximum AMC1 yield occurred at 24 hr for the RT-biocathode, which was markedly higher than that formed by the LT-biocathode (both closed and open circuits) (both at $p < 0.001$ level, $n = 18$ or $n = 3$). Meanwhile, the AMC12 yields (formed at 6 hr) between the RT- and LT-biocathode did not markedly differ. The maximum AMC1 yield for the biocathode at 10°C ($n = 18$) was significantly higher than that by the abiotic cathode at 10°C ($n = 3$, $p = 0.002$) (Fig. 3d).

AMC12 and NO formation first increased and then decreased for all the modes except the LT-open-circuit biocathode mode for AMC12 (24 hr was the time point for maximum yield). The amount of AMC12 formed with the LT-biocathode ($n = 18$) was obviously higher than that by the abiotic cathode ($n = 3$) (Fig. 3c). It is interesting to note that no NO was formed with the open-circuit biocathode, probably owing to the abiotic cathode surface that appeared on the biocathode, which allowed for the abiotic electrochemical CAP reduction under closed circuit mode (Fig. 3a). This was different from recent results obtained using different cathode electrode materials (carbon brush in this study vs. carbon cloth in a previous study) (Liang et al., 2013). In the test, the CAP-acetyl first accumulated and then was transformed in the exclusive microbial-catalyzed process (closed biocathode or open-circuit biocathode mode). More CAP-acetyl was accumulated by the open-circuit biocathode than by the closed biocathode, while the open-circuit LT-biocathode formed more CAP-acetyl than the open-circuit RT-biocathode within 24 hr ($n = 3$, $p = 0.014$) (Fig. 3b).

Microbial dehalogenation by catalyzing the cleavage of carbon-halogen bonds generally plays a key role in the detoxification of chlorinated compounds (Liang et al., 2012). The reduction of CAP to different products involved transfer of 2, 4, 6 and 8 electrons respectively for producing NO, the hydroxylamino intermediate, AMC12 and AMC1 from one CAP molecule. In this study, the biocathode dechloridation reaction generated significantly more AMC1 than did pure microbial or electrochemical dechloridation reactions (both at $p < 0.001$ level at 25°C and at $p < 0.001$ or $p = 0.002$ level, respectively, at 10°C) (Fig. 3d). These observations strongly indicated that cathodophilic microbes captured more electrons from the cathode electrode for CAP reduction and the biocathode-catalyzed process can enhance CAP reduction to
non-antibacterial products AMCl and AMCl2 (Liang et al., 2013; Smith et al., 2007). This study also illustrated that incorporation of the electrochemical catalytic process in the conventional anaerobic biological process can have a profound impact on the treatment of halogenated or nitroaromatic antibiotic micro-pollutants, giving products with no antibacterial activity products by a resilient and environmentally compatible biocathode BES that can cope with environments with fluctuating temperatures. Similar to the process of azo dye mineralization by incorporation of an aerobic bioreactor with the abiotic cathode/biocathode BES (breaking the azo linkage by cathodic reduction and mineralizing the produced aromatic amine by aerobic microbial catalysis) (Cui et al., 2012), this combined process would thoroughly mineralize products that are detoxified and have no antibacterial activity (AMCl2 and AMCl).

2.3. Anode potential, cathode potential and reaction current

The electricigens of the anode biofilm were enriched by inoculating with activated sludge at 25°C with a 0.5 V voltage supply. Acetate (1.68 g/L) and CAP (30 mg/L) served as the anode electron donor and cathode electron accepter, respectively. After 100 hr enrichment, the anode potential reached about −0.2 V (Fig. 4a). Subsequently, the cathode was inoculated with the CAP-reducing consortium five times to develop a cathode biofilm according to a previous study (Liang et al., 2013). In the start-up stage of the biocathode reactors, the reaction current of CAP reduction was decreased when the cathode electron acceptor CAP was nearly completely consumed. The cathode potential was kept at a stable level (approximately −0.7 V) during the start-up period (Fig. 4a).
After six batch-fed cycles were carried out, the operational temperature for the biocathode BES was switched from 25 to 10°C. The maximum current of CAP reduction at 10°C (0.31 ± 0.08 mA) was lower than that at 25°C (0.52 ± 0.06 mA) ($p < 0.001$). This occurrence should be attributable to the ability of the cathodophilic microbes in the biofilm to adapt to the applied temperature. Conversely, the same bioreactor group before temperature switchover had higher cathode current ($p = 0.045$) (Fig. 4b). These results indicated that low temperature stress decreased the activity of biocathodic CAP degradation. Although the anodophilic communities were enriched at 25°C, the current-producing performance was not affected at 10°C, which was different from the results of a recent study (Liu et al., 2013).

![SEM and CSLM micrographs](image)

**Fig. 6** – SEM (a–d) and CSLM (e–l) micrographs of cathode biofilms enriched at 10°C (two biological replicates: c, d, i–l) and 25°C (two biological replicates: a, b, e–h). Cathode biofilms were stained with SYTO 9 (green indicates active cathodophilic microbes) and propidium iodide (red notes dead cathodophilic microbes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![Cyclic voltammograms and Nyquist plots](image)

**Fig. 7** – Cyclic voltammograms of the biocathode (10 and 25°C) and abiotic cathode (a). Nyquist plots of the biocathode (10 and 25°C) and abiotic cathode (b).
2.4. Biocathodic glucose transformation

Microbial transformation of glucose (which served as the intracellular electron donor and growth carbon source for cathodophilic microbes) releases electrons, which could be used by cathodophilic microbes for the enhancement of CAP and nitrobenzene reduction (Liang et al., 2013; Sun et al., 2013; Wang et al., 2011). At 25°C, the biocathode reactors (divided into two groups) had the same trends for the efficiencies of glucose transformation and VFA as well as ethanol production (1–6 cycles in Fig. 5). Acetate was the main product in the glucose fermentation, followed by ethanol, isobutyric acid and propionic acid. When switching the operational temperature from 25 to 10°C, the acetate and ethanol yield of the LT-biocathode was decreased, which was consistent with the marked decrease in the glucose fermentation ability (7–13 cycles in Fig. 5a–c). The decreased activity of glucose metabolism by cathodophilic microbes at 10°C weakened the supply of intracellular electrons, aside from the extracellular electrons from the electrode to reduce CAP. The yields of propionic acid (except for cycle 8, p = 0.031) and isobutyric acid were not significantly changed (7–13 cycles in Fig. 5a and d).

2.5. Microbial role in the biocathodic CAP degradation

SEM photos of cathode biofilms revealed that populations of cathodophilic microbes declined with decreasing temperature (two biological replicates) (Fig. 6a–d). The live/dead staining CLSM analysis indicated that the RT-biocathode (Fig. 6e–h) had more attached cells than the LT-biocathode (Fig. 6i–l), correlating with the SEM results. The presence of electrochemically active microbes attached on the cathode at 10°C should explain why the LT-biocathode mode had significantly higher AMCl yield and $k_{\text{CAP}}$ than the abiotic cathode mode.

The CV tests showed that the RT-biocathode had the highest CAP reduction reaction current among the three operational modes. Decrease in operational temperature from 25 to 10°C apparently reduced the cathode current, but the value was still higher than that of the abiotic cathode. CV analysis highlighted the higher peak current and lower over-potentials for CAP reduction obtained with the biocathode compared with those for the abiotic cathode (Fig. 7a). This observation suggested that the cathode-attached microbes were involved in the electron transfer to CAP reduction, possibly via a direct (e.g., outer membrane c-type cytochromes) or indirect electron transfer mechanism (e.g., microbial produced mediators or periplasmic c-type cytochromes and hydrogenase networks) (Rosenbaum et al., 2011). However, these proposed electron transfer mechanisms within the complex biocathode microbial community need further investigation by functional metagenomics, metatranscriptomics and metaproteomics approaches. Restated, the CV tests confirmed the role of microbes on the biocathode on the noted CAP reduction activity.

EIS experiments were conducted to investigate the polarization resistance of electron transfer by the abiotic cathode, RT-biocathode and LT-biocathode, and the Nyquist plots of the three modes were fitted to the equivalent circuits (Fig. 7b). The polarization resistances for the RT-biocathode (9.6 Ω) or LT-biocathode (22.3 Ω) were lower than that for the abiotic cathode (36.4 Ω) (Fig. 7b). This finding demonstrated that attached growth of cathodophilic microbes would significantly enhance the rate of electron transfer for the biocathodic CAP reduction. Collectively, the CV, EIS, SEM and CLSM results suggested that the low polarization resistance and high cathode reaction current for the biocathode were attributable to the attached microbial growth on the cathode electrode. These tests also confirmed that electrochemically active cathodophilic microbes on the cathode significantly accelerated the electron transfer rate for the enhancement of biocathodic CAP degradation.

3. Conclusions

Biocathode-catalyzed activity for CAP degradation was obviously decreased when the temperature dropped by 15°C, while the biocathode BES had significantly higher $k_{\text{CAP}}$, nitro-group reduction and dechloridation efficiency compared with those of the abiotic cathode BES. Electrochemically active cathodophilic microbes on the cathode lowered polarization resistance and over-potentials and finally accelerated the electron transfer rate for the biocathodic CAP degradation in the BES. CAP was efficiently degraded by the biocathode BES under large temperature switchover. Such a resilient and efficient process has potential use in CAP-containing wastewater treatment.

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