Inactivation of antibiotic-resistant bacteria and antibiotic resistance genes by electrochemical oxidation/electro-Fenton process

Lei Chen, Zhi Zhou, Chaofeng Shen and Yilu Xu

ABSTRACT

Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment are of great concern due to their potential risk to human health. The effluents from wastewater treatment plants and livestock production are major sources of ARB and ARGs. Chlorination, UV irradiation, and ozone disinfection cannot remove ARGs completely. In this study, the potential of electrochemical oxidation and electro-Fenton processes as alternative treatment technologies for inactivation of ARB and ARGs in both intracellular and extracellular forms was evaluated. Results showed that the electrochemical oxidation process was effective for the inactivation of selected ARB but not for the removal of intracellular ARGs or extracellular ARGs. The electro-Fenton process was more effective for the removal of both intracellular and extracellular ARGs. The removal efficiency after 120 min of electro-Fenton treatment under 21.42 mA/cm² was 3.8 logs for intracellular tetA, 4.1 logs for intracellular ampC, 5.2 logs for extracellular tetA, and 4.8 logs for extracellular ampC, respectively in the presence of 1.0 mmol/L Fe²⁺. It is suggested that electrochemical oxidation is an effective disinfection method for ARB and the electro-Fenton process is a promising technology for the removal of both intracellular and extracellular ARGs in wastewater.

Key words | antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), electrochemical oxidation, electro-Fenton process, wastewater treatment

HIGHLIGHTS

- Electrochemical oxidation process was effective for the inactivation of selected ARB but not for ARGs.
- The electro-Fenton process is an effective method for the removal of ARB and both intracellular and extracellular ARGs.

INTRODUCTION

The excessive use of various antibiotics led to the widespread distribution of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in various environment matrices, such as water, sludge, soil, sediment, etc. (Auguet et al. 2017; Qiao et al. 2018; Cheng et al. 2019). This resistance reduces the efficacy of antibiotics for infectious diseases, which causes over 23,000 deaths per year in the USA, nearly 25,000 deaths per year in Europe and even more in less-developed countries (WHO 2014; Vikesland et al. 2017). It has become a global issue for human and animal health. The antibiotic resistance can be disseminated by sharing ARGs among microorganisms through horizontal gene transfer (Sharma et al. 2016). Plasmids, integrons, and transposons are the mobile genetic elements often involved in the ARG sharing processes (Sharma et al. 2016). ARGs can persist in
the environment even after the bacteria are dead (Dodd 2012). Both intracellular and extracellular ARGs can readily adapt into new hosts (Pruden 2014). The effluents from wastewater treatment plants (WWTPs) and livestock production, often with high concentrations of ARGs, are considered to be significant sources of ARB and ARGs in the environment (Auguet et al. 2017).

The conventional disinfection processes such as chlorination, UV irradiation, and ozone oxidation in water and wastewater treatment have been proven to inactivate ARB effectively (Sharma et al. 2016). However, most of the ARGs remained even when the ARB are completely inactivated in the disinfection processes (Dodd 2012). Sometimes the disinfection processes may destroy bacterial DNA or the cellular structure, but ARGs may still exist in the cell debris, and the extracellular ARGs can still pose a continuing risk (Czekalski et al. 2016). Recently, technologies for removal of intracellular ARGs have been largely investigated, including enhanced disinfection, constructed wetland, and advanced oxidation process (AOPs). A study by Zhang et al. (2015) showed a positive relationship between the inactivation of ARGs and the dosage of chlorine and contact time. They also pointed out that sequential UV/chlorination could enhance the inactivation significantly. In order to decrease both ARB and ARGs effectively, a dose of chlorine as high as 50 mg/L or a dose of 5 mg/L ozone is required (Oh et al. 2016). As the dosage of UV irradiation increased, the abundance of ARGs decreased exponentially (Zheng et al. 2017). High doses of UV irradiation (>10 mJ/cm²) decreased ARB and ARGs significantly but largely increased the frequency of ARGs transfer simultaneously for the higher pressure (Guo et al. 2013). Constructed wetlands, especially those with a surface flow pattern, have shown good ARGs reduction efficiencies; but the risk of increased ARGs transfer still remained (Huang et al. 2013). Therefore, new alternative processes with high removal efficiency and low risk of ARGs transfer are strongly needed. AOPs such as Fenton oxidation, TiO₂ photocatalysis, and UV/H₂O₂ have recently shown high potential to inactivate ARB and ARGs. The hydroxyl radical (·OH) in the Fenton process and UV/H₂O₂ treatment could remove ARGs effectively (2.3–3.8 logs of reduction) and the Fenton process performed better than UV/H₂O₂ treatment (Zhang et al. 2016; Yoon et al. 2017). The study by Guo et al. (2017) demonstrated that photocatalysis by TiO₂ could reduce ARB by 4.5–5.8 logs and ARGs by 4.7–5.8 logs. AOPs are promising ways for ARB and ARGs removal because of their high removal efficiency. Nevertheless, there are still some gaps of knowledge. For example, although electrochemical disinfection has been widely used in the inactivation of various bacteria, viruses, and microalgae, its potential on ARGs removal has not been studied in depth (Rahmani et al. 2019). All the above studies have focused on the removal of intracellular ARGs; the removal efficiencies for extracellular ARGs are still unknown.

The aim of this study is to investigate the capacities of electrochemical oxidation and electro-Fenton processes for inactivation of ARB and ARGs. Two representative ARB (Escherichia coli SMS-3-5 and Pseudomonas aeruginosa 01) and two targeted ARGs hosted in the two bacteria (tetA and ampC) were studied. The results suggest a potential alternative method for effective inactivation of ARB and ARGs in wastewater.

**METHODS**

**Preparation of bacteria and quantification**

E. coli SMS-3-5 (ATCC BAA-1743) and P. aeruginosa 01 (ATCC 47085), which are both multi-drug resistant, were obtained from American Type Culture Collection (ATCC). In this study the ARGs targeted for E. coli SMS-3-5 and P. aeruginosa 01 are tetA and ampC, respectively. Detailed information of these two bacteria was described by McKinney & Pruden (2012). Bacteria were first recovered following the User Instruction Manual in the sterile lab and then stored in Luria–Bertani broth (LB) with 30% glycerinum at -80 °C after three generations of incubation. The two bacteria were prepared separately by spiking 1 mL of frozen stock culture into 100 mL of LB broth. Cultures were grown at 37 °C in an incubator shaker to approximately mid-exponential phase, which provided a relatively homogeneous culture to be compared across studies (McKinney & Pruden 2012). The cell density was determined by measuring the optical density (OD₆₀₀) using a spectrophotometer. The heterotrophic plate count method was used to determine the quantity of each ARB in the reaction solution (Gao et al. 2012). Fungal growth was inhibited by adding 200 mg/L cycloheximide (Zheng et al. 2017). Ten-fold serial dilutions were prepared from each sample and then plated. After incubation under dark conditions at 37 °C for 24 h, the colony-forming unit on each plate was counted manually.

**DNA extraction and real-time quantitative polymerase chain reaction (qPCR)**

In order to prepare the reaction solutions containing only free extracellular DNA for electrochemical experiments,
the extracellular DNA was extracted from a large amount of bacteria cells via phenol/chloroform method, which is a traditional and effective way to obtain large quantities of DNA (Sambrook & Russell 2001). The concentration and purity of extracted DNA were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) with the detection limit of 2 ng/L. The ratio of A260/280 was 1.58–1.76, which could be accepted as pure DNA (Sambrook & Russell 2001). For extraction of intracellular DNA in the samples collected during the electrochemical experiments, the sample was centrifuged for 10 min at 10,000 × g before the bacterial pellet was extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories, USA), which was more convenient than the traditional phenol/chloroform method. Purity of extracted intracellular DNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). For extraction of extracellular DNA in the samples collected during electrochemical experiments, 0.6 mL of isopropanol was first added into the sample. The precipitate was collected and cleaned with 70% alcohol before it was dissolved in the Tris-EDTA (TE) buffer (Sambrook & Russell 2001).

Table 1 | Primers used in this study

<table>
<thead>
<tr>
<th>ARB</th>
<th>Target genes</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>tet(A)</td>
<td>F</td>
<td>GCTACATCCTGCTTGCGTTC</td>
<td>60</td>
<td>210</td>
<td>Zheng et al. (2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CATAGATCGCCGTAAGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>ampC 01</td>
<td>F</td>
<td>CCTCTTGCTTCCACAATTGCT</td>
<td>58</td>
<td>189</td>
<td>Shi et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>ACAACGTGTTGCTGTGACG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Both the intracellular and extracellular ARGs in samples were quantified using SYBR Green I qPCR on a real-time PCR. The primers, annealing temperature, and amplification size are listed in Table 1. qPCR standard was prepared according to the description by Guo et al. (2017). qPCR reactions were conducted in 96 well plates. The final volume of mixture in each well was 20 μL containing 10 μL of iTaq Universal SYBR Green Supermix (Bio-Rad, USA), 0.2 μM of each primer, 2 μL of template DNA, and 7.2 μL of ultrapure water. The qPCR amplification and quantification processes were carried out on a StepOnePlus real-time PCR system (Applied Biosystems, USA) with the following protocol: 2 min at 95 °C, followed by 40 cycles at 95 °C for 5 s, 10 s at annealing temperature, 15 s at 72 °C, and a final melt curve stage for specificity verification. Each reaction was run in triplicate. qPCR efficiencies were in the range of 87%–112% with R² values more than 0.990 for all calibration curves.

### Electrochemical experiments

Electrochemical experiments were conducted in an undivided cylindrical glass cell containing 300 mL of reaction solution with a platinum electrode as the anode, graphite felt electrode as the cathode, and a silver/silver chloride electrode as the reference electrode. All the electrodes were connected with a DC power supply. The active surface (immersed) was 7 × 5 cm. The reaction solution was prepared using filtered (through a 0.22 μm membrane) and sterilized wastewater. The wastewater was obtained from a WWTP after secondary clarification with the pH of 7.0–7.2, chemical oxygen demand (CODₜₜ) of 35 mg/L, total nitrogen of 13 mg/L, and ammonia nitrogen (NH₃–N) of 5 mg/L. The reaction solution contained about 2.5 × 10¹¹ cells/L (OD₆₀₀ of 0.25) or 100 ng/L of extracellular DNA. Then, 100 mmol/L of Na₂SO₄ was added as the electrolyte. The solution was stirred using a magnetic bar. Compressed air was bubbled into the cell continuously at a flow rate of 0.6 L/min. The initial pH of the solution was about 7.0 and different current densities (7.14, 14.28, or 21.42 mA/cm²) were applied during electrochemical oxidation processes. In the electro-Fenton process different amounts of ferrous ions (0.5, 1.0, or 2.0 mmol/L) were introduced into the cell and the effect of initial pH, which was modified by adding H₂SO₄ or NaOH, was also investigated under a constant current density of 21.42 mA/cm². As the pH of reaction solution was slowly increased during the electro-Fenton process, H₂SO₄ was added to adjust the pH. All electrochemical experiments were performed in triplicate at 25 °C. Control experiments with no current applied were carried out simultaneously. Samples of 1 mL were collected periodically to determine the quantities of ARB and intracellular and extracellular ARGs.

### Data analysis

Gene copy numbers were log-transformed to normalize the distributions prior to statistical analysis. The removal of ARGs was calculated as log removal (log C/C₀), where C₀
was the abundance of ARGs at the initial time (T₀), and C was the abundance of ARGs at each sampling time (T). Statistical analysis was conducted using Statistical Package for the Social Sciences 16.0 for Windows (USA). One-way analysis of variance was applied to detect significant differences and the statistical significance was accepted at \( p < 0.05 \).

RESULTS AND DISCUSSION

Inactivation of ARB and ARGs by the electrochemical oxidation process

Inactivation efficiencies of *E. coli* SMS-3-5 and *P. aeruginosa* 01 under different current densities are shown in Figure 1. When no current was applied, both types of bacteria were hardly inactivated. When current was applied, both types of bacteria were inactivated to some degree during the electrochemical oxidation process. The cell density of both ARB decreased very slowly during the initial 60 min, which might be due to the defensiveness of antioxidative enzymes against the oxidative stress (Sun *et al.* 2014). The inactivation rate then increased dramatically. Increasing current density from 7.14 mA/cm² to 21.42 mA/cm² increased the inactivation efficiency of both bacteria significantly. After 120 min of treatment, 1.7 logs of reduction for *E. coli* SMS-3-5 and 2.1 logs of reduction for *P. aeruginosa* 01 were achieved under the current density of 21.42 mA/cm². Moreover, the inactivation efficiency of *E. coli* SMS-3-5 was lower than that of *P. aeruginosa* 01 under the same current density, suggesting that *E. coli* SMS-3-5 was more resistant to electrochemical oxidation than *P. aeruginosa* 01.

The electrochemical process is considered a promising environmentally friendly disinfection method for water and treated wastewater. It has been successfully applied in the inactivation of various bacteria, viruses, and microalgae (Li *et al.* 2011). During the electrochemical treatment, H₂O₂ was generated at the cathode. This either directly attacked the cellular membrane, reducing the viability of cells, or diffused into cells where it reacted with intracellular biomolecules and thus inactivated the cells (Valero *et al.* 2017). The concentration of H₂O₂ in the solution was determined, which was 0.24 mmol/L under 7.14 mA/cm², 0.51 mmol/L under 14.8 mA/cm², and 0.53 mmol/L under 21.42 mA/cm². At the low current intensity, when the concentration of H₂O₂ was low, the bacteria seemed to have resistance to electrochemical oxidation. Increased current density could produce more H₂O₂ and result in a higher inactivation of ARB, which was in agreement with those found in other studies of *E. coli* inactivation by the electrocoagulation process and photocatalytic system (Li *et al.* 2011).
The results from the study of antibiotic-resistant *E. coli* S1-23 by Jiang et al. (2017) showed no obvious reduction in cell density after 14 h of electrochemical treatment. The explanation might be that the flow-through thin-layer electrochemical reactor was used in their study and the contact time might have been too short.

The removal of intracellular *tetA* and *ampC* is shown in Figure 2. Even when a high current density of 21.42 mA/cm² was applied, only 0.58 logs and 0.72 logs of intracellular ARG removal was achieved for *tetA* and *ampC*, respectively, after 120 min of electrochemical oxidation treatment. It seems that the electrochemical oxidation process was not effective for the removal of intracellular ARGs. By contrast, extracellular ARGs could be removed more effectively by electrochemical oxidation treatment. As shown in Figure 3, after 120 min of treatment, 1.8 logs and 1.5 logs of extracellular ARGs removal was achieved for *tetA* and *ampC*, respectively under 21.42 mA/cm². Concentrations of both extracellular ARGs decreased quickly from the start without a lag phase as the electrochemically produced H₂O₂ directly oxidized the dissolved DNA. The concentration of H₂O₂ in the solution under 21.42 mA/cm² was about 0.82 mmol/L. The main mechanism for the removal of extracellular ARGs might be the direct oxidation by the H₂O₂ generated. The removal efficiency of extracellular *tetA* was slightly higher than that of extracellular *ampC*, which was inconsistent with the findings for ARB and intracellular ARGs.

**Removal of ARB and ARGs by the electro-Fenton process**

As the target intracellular ARGs were hardly inactivated by the electrochemical oxidation process and the extracellular ARGs could only be slightly removed, removal efficiency by the electro-Fenton process was evaluated. Different amounts of Fe²⁺ were added to the solutions under the constant current density of 21.42 mA/cm². Inactivation efficiencies of ARB in the presence of different amount of Fe²⁺ are shown in Figure 4. When Fe²⁺ was added, the inactivation of ARB was significantly enhanced, reaching 4.3 logs and 4.7 logs of reduction for *E. coli* SMS-3-5 and *P. aeruginosa* 01, respectively, in the presence of 1.0 mmol/L Fe²⁺. The influence of Fe²⁺ on the removal of intracellular ARGs and extracellular ARGs at pH 3.5 is shown in Figures 5 and 6, respectively. Compared with the electrochemical oxidation process, removal of both intracellular and extracellular ARGs by electro-Fenton treatment was promoted. Increasing Fe²⁺ concentration from 0.5 mmol/L to 1.0 mmol/L increased the removal efficiency of extracellular ARGs.
efficiency of both intracellular and extracellular ARGs significantly, particularly for intracellular ARGs. However, when the concentration of Fe$^{2+}$ was increased to 2.0 mmol/L, the removal efficiency of both intracellular and extracellular ARGs decreased. The highest removal efficiency after 120 min of electro-Fenton treatment under 21.42 mA/cm$^2$ was 3.8 logs for intracellular tetA, 4.1 logs for intracellular ampC, 5.2 logs for extracellular tetA, and 4.8 logs for extracellular ampC, respectively, when 1.0 mmol/L Fe$^{2+}$ was added.
The Fenton process has shown favorable effects on the degradation of various organic compounds due to the rapid generation of reactive oxygen species such as hydroxyl radicals (•OH), superoxide radicals (O$_2^-$), and singlet oxygen (1O$_2$) by the decomposition of H$_2$O$_2$ catalyzed by Fe$^{2+}$, where •OH was the main species that played the important part (Babuponnusami & Muthukumar 2014). During the electro-Fenton process, H$_2$O$_2$ is continuously electro-generated in situ and reactive oxygen species, especially •OH, are produced with the addition of an Fe$^{2+}$. 

![Figure 5](image1.png) **Figure 5** | Effect of Fe$^{2+}$ concentration on the removal of intracellular ARGs by electro-Fenton process (Current density: 21.42 mA/cm$^2$; pH = 3.5).

![Figure 6](image2.png) **Figure 6** | Effect of Fe$^{2+}$ concentration on the removal of extracellular ARGs by electro-Fenton process (Current density: 21.42 mA/cm$^2$; pH = 3.5).
The -OH are primarily responsible for the inactivation of ARB and both intracellular and extracellular ARGs during the electro-Fenton process with Fe$^{2+}$ added and at a pH of 3.5 in the present study. The -OH first destroyed the cell structure and then penetrated into the cells, oxidizing the intracellular DNA molecule or directly oxidizing the extracellular DNA molecule.

The concentration of H$_2$O$_2$ and Fe$^{2+}$ and solution pH have been reported to be the main factors that determined the efficiency of the Fenton process (Trapido et al. 2009). Compared with the Fenton process, the main advantages of the electro-Fenton process are that it has less energy consumption, less waste production, and easier handling of H$_2$O$_2$ (Valero et al. 2017). It is well known that higher concentrations of Fe$^{2+}$ could yield higher rates of reaction; nevertheless, higher concentrations of Fe$^{2+}$ will result in more consumption of H$_2$O$_2$ due to the scavenging effect, which is not good for the yield of -OH (Babuponnusami & Muthukumar 2014). This may explain the observation that as the Fe$^{2+}$ concentration increased, the removal efficiency of ARGs first increased and achieved the highest at 1.0 mmol/L of Fe$^{2+}$, and then decreased.

There was also a lag phase in the decrease of intracellular ARGs, as shown in Figure 5. It might be the viability of the bacterial cells during the first period of electro-Fenton treatment, which maintains the cell structural integrity. However, the lag phase for intracellular ARGs in the electro-Fenton process was about 30 min, which was shorter than that in the electrochemical oxidation process. Contrarily, there was no lag phase in the decrease of extracellular ARGs. The removal efficiency of extracellular _tetA_ was higher than that of extracellular _ampC_ in the electro-Fenton process, which was in accordance with that in the electrochemical oxidation process. On the whole, both intracellular and extracellular ARGs could be removed effectively by the electro-Fenton process; however, the degradation products were not detected in the present study. In a further study, the degradation products will be detected in case some other pollutant might be generated at the same time.

In order to determine the effect of initial pH on the removal of ARGs during the electro-Fenton process, experiments were performed under a current density of 21.42 mA/cm$^2$ with 1.0 mmol/L of Fe$^{2+}$ added. The removal of intracellular ARGs and extracellular ARGs after 120 min of treatment at different pH is shown in Figure 7. When the pH value increased from 3.0 to 7.0, the removal of intracellular ARGs first increased and then decreased, with the maximum removal of both intracellular and extracellular ARGs at pH 3.5. A previous study on ARGs inactivation.

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**Figure 7** | Effect of initial pH on the removal of ARGs after 120 min of electro-Fenton treatment (Current density: 21.42 mA/cm$^2$, Fe$^{2+}$ concentration: 1.0 mmol/L).
in wastewater effluent by the Fenton process revealed that the maximum removal of various ARGs was achieved at pH 3.5, which was in accordance with the results of our study (Zhang et al. 2019). The Fenton oxidation process is often controlled within the pH of 3.0–3.5 because less amount of the ·OH radical is produced due to the formation of Fe²⁺ complexes with water at a lower pH (<2.5) or the precipitation of ferric oxyhydroxides at a higher pH (>4.0) (Gogate & Pandit 2004). The removal of extracellular ARGs during the Fenton process might be due to the direct oxidation by ·OH produced at pH 3.5 with the presence of Fe²⁺. Compared with those under acidic conditions, the removal efficiency of both intracellular and extracellular ARGs under neutral condition was relatively lower, which was only between 2.2 and 3.3 logs. Water and urban wastewater pH is typically in the range of 7.0 to 8.0, which means that ARGs could only be partially removed under the original pH condition without acidification.

Comparison of the electro-Fenton process with other AOPs technologies

It has been proven that advanced oxidation processes are more effective on the inactivation of ARGs than conventional disinfection methods such as chlorination, UV, and ozonation. The study by Zhang et al. (2016) showed that the maximum removal of ARGs by the UV/H₂O₂ process and the Fenton process under the optimal condition was 2.8–3.5 logs and 2.6–3.8 logs; while another study reported that a reduction of 5.2 logs intracellular mecA, 3.3 logs extracellular mecA, 4.4 logs intracellular ampC, and 2.6 logs extracellular ampC were achieved by UV/H₂O₂/TiO₂ photocatalysis (Guo et al. 2017), as shown in Table 2. It seems that the electro-Fenton process is a promising technology for removal of both intracellular and extracellular ARGs in wastewater.

CONCLUSIONS

During the electrochemical oxidation process, two ARB *E. coli* SMS-3-5 and *P. aeruginosa* 01 were both inactivated effectively, but the targeted intracellular ARGs were hardly removed and the extracellular ARGs could only be slightly removed with the reduction of 1.5–1.8 logs under the current density of 21.42 mA/cm². When Fe²⁺ was added, the removal of both intracellular and extracellular ARGs was significantly promoted. Under the current density of 21.42 mA/cm² and initial pH of 3.5 with 1.0 mmol/L of Fe²⁺ added, the removal efficiency of ARGs after 120 min of treatment achieved 3.8–4.1 logs for intracellular ARGs and 4.8–5.2 logs for extracellular ARGs. The results suggest that electrochemical oxidation is an effective disinfection method for ARB and the electro-Fenton process is a promising technology for the removal of both intracellular and extracellular ARGs in wastewater.

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