Electrochemical oxidation of fluoroquinolone antibiotics: mechanism, residual antibacterial activity and toxicity change

Linyan Zhu[a,b], Beatrix Santiago-Schübel[a], Hongxia Xiao[b], Henner Hollert[b,c,d,e], Stephan Kueppers[a]

[a] Research Center Jülich, ZEA-3, Jülich 52425, Germany

[b] Department of Ecosystem Analysis, Institute for Environmental Research, ABBt- Aachen Biology and Biotechnology, RWTH Aachen University, Aachen 52074, Germany

[c] College of Resources and Environmental Science, Chongqing University, 1 Tiansheng Road Beibei, Chongqing 400715, People's Republic of China

[d] Key Laboratory of Yangtze Water Environment, Ministry of Education, Tongji University, Siping Road 1239, Shanghai 200092, People's Republic of China

[e] State Key Laboratory of Pollution Control & Resource Reuse, School of the Environment, Nanjing University, Xianlin Avenue 163, Nanjing 210046, People's Republic of China

*Corresponding author:

Stephan Kueppers

Tel.: +49 2461612766; fax: +49 2461612560.

E-mail address: s.kueppers@fz-juelich.de

Abstract:

In this paper, we studied the electrochemical oxidation mechanisms of three typical fluoroquinolone antibiotics (FQs), and investigated residual antibacterial activity and toxicity changes after oxidation processes. Electrochemistry coupled to mass spectrometry (EC-MS) was used to study the oxidation processes of ciprofloxacin (CIP), norfloxacin (NOR) and ofloxacin (OFL). Eight oxidation products for each parent compound were identified and their chemical structures were elucidated. The transformation trend of each product, with the continuous increase of voltage from 0 to 3000 mV, was recorded by online EC-MS. The oxidation pathways were proposed based on the structural information and transformation trends of oxidation products. We found the oxidation mechanisms of FQs consisted of the hydroxylation and cleavage of piperazinyl ring via reactions hydroxyl radicals, while the fluoroquinolone core remained intact. The antibacterial activity of the parent compounds and their oxidation mixtures was estimated using zone inhibition tests for gram-negative bacteria *Salmonella typhimurium*. It was found that the oxidation mixtures of CIP and NOR retained the antibacterial properties with lower activity compared to their parent compounds, while the antibacterial activity of OFL was almost eliminated after oxidation. Furthermore, the toxicity of the three FQs and their oxidation mixtures were evaluated by using algal growth inhibition test (*Desmodesmus subspicatus*). The median effective concentration (EC_{50}) values for the algal inhibition tests were calculated for the end point of growth rate. The toxicity of CIP and NOR to green algae after electrochemical oxidation, remained unchanged, while that of OFL significantly increased. The results presented in this paper contribute to an understanding of the electrochemical oxidation mechanisms of FQs, and highlight the potential environmental risks of FQs after electrochemical oxidation processes.

Keywords: Fluoroquinolone antibiotics, electrochemistry-mass spectrometry, antibacterial activity, toxicity change

1. Introduction

Fluoroquinolone antibiotics (FQs) are the most widely used group of antibiotics in the treatment of respiratory and bacterial infections, due to their board-spectrum activity against bacteria. FQs have been detected in wastewater treatment plant (WWTP) effluents (Kostich et al., 2014), surface water (Zhang et al., 2014) and in various environment matrixes (Kümmerer, 2009) with concentrations from ng/L to μg/L level.

The presence of FQs in the environment can induce adverse effects on organisms and human beings in the long term, even at trace concentrations (Isidori et al., 2005 and Johansson et al., 2014). First, continuous release of FQs into the aquatic environment may induce antibiotic resistance in native bacterial population (Bos et al., 2015). Resistance has the potential to adversely affect the health of aquatic and terrestrial organisms including humans (Bengtsson-Palme and Larsson, 2015 and Kümmerer, 2009). Secondly, the presence of FQs raises great concern about their toxicity in the environment. Robinson et al. (2005) conducted a study which found seven FQs exhibiting selective toxicity to five aquatic organisms. The combined toxicity of FQs and other antibiotics was investigated by González-Pleiter et al. (2013) on cyanobacterium and green alga, finding that strong synergism between these compounds observable in both organisms. Therefore, it is of great importance to effectively eliminate FQs from wastewater.

Advanced oxidation processes (AOPs) include a large variety of methods, such as ozone based processes, photolysis and photocatalysis processes and Fenton reaction based processes, which can effectively combine with conventional processes to remove resistant pharmaceuticals. Among them, electrochemical advanced oxidation processes (EAOPs) have recently received

increasing attention due to their high-energy efficiency, versatility, and safety (Sirés and Brillas, 2012). The simplest and most popular EAOP is anodic oxidation with electrogenerated hydroxyl radicals (·OH) on boron-doped diamond (BDD) electrodes (Eq. (1)). BDD electrodes are preferred for water remediation since they can generate high amounts of weakly physisorbed hydroxyl radicals (Marselli et al., 2003 and Moreira et al., 2014), which enhance the removal of organic chemicals.

$$BDD + H_2O \rightarrow BDD(\cdot OH)_{ads} + H^+ + e^- \quad (1)$$

Numerous investigations have been conducted on the removal of FQs by EAOPs, most of which were focused on the optimization of reaction conditions, oxidation kinetics, and efficiency (Carlesi Jara et al., 2007, Guinea et al., 2009, Guinea et al., 2010 and Yahya et al., 2014). However, little attention has been paid to the understanding of oxidation mechanisms of FQs during EAOPs.

Online or offline coupling electrochemistry with mass spectrometry (EC-MS) was first used to study the redox reactions of biomolecules (Hambitzer and Heitbaum, 1986 and Volk et al., 1989) and simulate drug metabolism (Karst, 2004). In our previous studies (Chen et al., 2012, Chen et al., 2014 and Hoffmann et al., 2011), EC-MS has been shown to be a reliable and rapid laboratory tool to investigate the oxidative mechanisms of organic pollutants in the environment and water treatment processes. In particular, the online monitoring of electrochemical oxidation processes has the advantage of directly detecting highly reactive and short-lived intermediates without a time delay. Therefore, we applied this approach to investigate oxidative mechanisms and identify oxidation products of FQs by electrochemical oxidation.

However, the abatement of the FQs during electrochemical oxidation can lead to the formation of various oxidation intermediates and products. There is a great possibility that electrochemical oxidation products also retain the biological effects of their parent compounds, and even develop new undesired bio-effects. In earlier studies (De Bel et al., 2009, Michael et al., 2010 and Vasquez et al., 2013), residual antibacterial activity and toxicity changes in FQs after other oxidation processes, such as ozonation, UV treatment, and photocatalysis have been reported. Therefore, the issue cannot merely be addressed by elucidating the structures of oxidation products. Moreover, the toxicological effects of treated solutions arising from a mixture of residual parent compounds and their oxidation products should be evaluated.

The objective of the present paper is to study electrochemical oxidation mechanisms of FQs and evaluate the antibacterial activity and toxicity of the FQs and their reaction mixtures. Three typical FQs (Fig.1), ciprofloxacin (CIP), norfloxacin (NOR) and ofloxacin (OFL), which are the most frequently detected in WWTPs and natural water (Kostich et al., 2014, Zhang et al., 2014 and Van Doorslaer et al., 2014), were selected to conduct this study. We identified oxidation products and proposed oxidation pathways of the three FQs using EC-MS. A bulk EC cell for rapid electro-synthesis, introduced in our latest work (Zhu et al., 2015), was used to prepare mg quantities of the reaction mixtures for biological tests. The antibacterial activity of the FQs and their oxidation mixtures was assessed using a typical zone inhibition test with gram-negative bacteria, *Salmonella typhimurium*, as a reference bacterium. The toxicity change of the FQs during electrochemical oxidation processes was investigated using algal growth inhibition tests with green algae (*Desmodesmus subspicatus*).

2. Materials and methods

2.1 Chemicals and regents

All solvents (chromatographic grade) and chemicals (analytical grade) were used as received from the commercial suppliers. Ciprofloxacin (99%, CAS 85721-33-1), norfloxacin (99%, CAS 70458-96-7), and ofloxacin (99%, CAS 82419-36-1) were purchased from Sigma-Aldrich. Ammonium acetate (p.a.), methanol (LiChrosolv purity), and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid was obtained from ROMIL (Cambridge, UK). High purity water (18.2 MΩ·cm) was produced by a MilliQ plus 185 (Millipore, Molsheim, France).

2.2 Online EC-MS setup

2.2.1 Electrochemical reactions

A commercial EC reactor (Antec Leyden, The Netherlands) with a built-in platinum counter electrode and Roxy potentiosat was set up as reported in previous investigations (Chen et al., 2012 and Hoffmann et al., 2011).

The electrochemical reactions were conducted in a flow-through “ReactorCell” (Antec Leyden, The Netherlands) containing a working electrode and a pH-dependent HyREF electrode for reference. In this study, a BDD working electrode was used for oxidation. Each reaction solution was composed of 50 µM of the parent compound with a 10 mM ammonium acetate buffer, in a mixture of methanol and water (2:3) containing 0.1% formic acid. The reaction solution was pumped through the EC cell at a constant flow rate of 10 µL/min. The residence time of the solution at the working electrode was approximately 3s. A potential ramp at a scan rate of 10

mV/s was applied to record the dynamic transformation processes of target ions. The mass spectra of the FQs at different reaction voltages were recorded by applying constant voltages to the EC cell. All reactions were conducted at a constant temperature (25°C) and repeated in triplicate to ensure the stability of the system and minimize bias and random errors.

2.2.2 Mass spectrometry conditions

ESI-MS experiments were carried out on a QTRAP 2000 (ABSciex, Darmstadt, Germany) and a high-resolution Fourier Transform Ion Cyclotron Resonance mass spectrometer (ESI-FTICR-MS) Ultra (ThermoFisher Scientific, San Jose, CA, USA), respectively. The settings of the method were performed as given in detail by Chen et al. (2012) and Hoffmann et al. (2011).

2.3 Preparation of reaction mixture for toxicity testing

Scaling up reactions were conducted in an offline "SynthesisCell" (Antec Leyden, The Netherlands) following the work-up method introduced in our previous work (Zhu et al., 2015). An 80 mL solution containing 8 mg of a parent compound, and 10 mM ammonium acetate with 0.1% formic acid, was oxidized in the "synthesis cell" for approximately 2 h. The reaction solution was stirred throughout this period by a magnetic stirrer. 400 mL of the reaction solution was collected by repeating this reaction five times. The solvent of the reaction mixture was removed by a SpeedVac system (Thermo Fisher Scientific, USA). The samples were dissolved in water and the pH of the sample solution was adjusted to 3. Based on the structures and properties of FQs and their oxidation products, a CHROMABOND® HR-XCW column (6mL/500 mg, Macherey and Nagel, Düren, Germany) was selected as the solid phase for solid phase extraction (SPE). Each SPE column was conditioned twice with 40 mL MeOH and then 10 mL H_2O (pH=3). An 80 mL sample solution was loaded onto the columns at a flow rate 4-5 mL/min.

Each column was then washed twice with 10 mL water, and the components of the sample were eluted with 1 mL MeOH/ACN/Formic acid (20/75/5) five times. Finally, the eluate was dried using the SpeedVac.

The F^- concentrations in the reaction solutions of CIP, NOR and OFL were measured by an ion chromatography system (ICS-3000, Dionex, USA). An IonPac AS23 Anion-Exchange Column (4 x 250 mm, Dionex, USA) was used for separation. The column temperature was set to 30 °C. The mobile phase was composed of 4.5 mM of Na_2CO_3/0.8 mM of $NaHCO_3$, and the flow rate was set at 1.0 mL/min.

The final oxidation mixtures of the three FQs, named Ciprofloxacin Product (CP) mixture, Norfloxacin Product (NP) mixture and Ofloxacin Product (OP) mixture, were analyzed by an Agilent1100 DAD-HPLC system (Agilent Technologies, Waldbronn, Germany) interfaced with a Q-TRAP 4000 (ABSciex, Darmstadt, Germany). A C18 Eclipse Plus column (100 x 4.6 mm, 2.6 µm particle size, Agilent Technologies, Waldbronn, Germany) was used for separation. The column temperature was set to 30 °C. The binary mobile phase consisted of (A) H_2O with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. A 17 min gradient was used as follows: 0-2 min 90% A isocratic, 2-10 min 10-90% B linear, 10-13min 90% B isocratic, 13-13.10 min 10-90% A linear, 13.10-17 min 90% A isocratic at a flow rate of 800 µL/min. The components were analyzed in the multiple reaction monitoring (MRM) mode (CIP: 332->288, NOR: 320->276, OFL: 362->318).

2.4 Antibacterial activity test

169 An inhibition zone test was carried out according to the Disk Diffusion Test Methodology by
170 The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Matuschek et al.,
171 2014) with slight modifications.

172 *Salmonella typhimurium*, as a typical Enterobacteriaceae, was selected as the test bacteria. *S.*
173 *typhimurium* was cultured overnight in Oxoid Nutrient Broth no. 2 (Sigma-Aldrich), at 37 ± 1°C,
174 in an incubator shaking at 150 rpm for 8-10 h. Densities of the overnight inoculum were
175 computed in formazine attenuation units (FAU) by relating measured optical densities (λ = 595
176 nm) (Infinite 200, Tecan, Crailsheim, Germany). 100 µL of individual stock cultures were spread
177 on nutrient agar plates, containing 15 g/L agar, 7.5 g/L yeast extract, 7.5 g/L casein Peptone and
178 5.0 g/L NaCl (Merck KGaA, Damstadt, Germany). Filter papers (Ø: 8 mm) spiked with 10 µL of
179 an aqueous sample solution were placed on the plates. Subsequently, the plates were incubated at
180 37±2°C for 16-20 hours. After incubation, the inhibition zone diameters were measured to the
181 nearest millimeter. Inhibition zone diameter is the criteria indicating the antibacterial activity.
182 The statistical difference between the parent compound and its oxidation mixture at the same
183 exposure concentration was calculated by a t-test ($p < 0.05$). The statistical differences among
184 different exposure concentrations of the same sample were determined by a one-way ANOVA
185 followed by Student-Newman-Keuls multiple comparison test ($p < 0.001$).

186 **2.5 Algal growth inhibition assay**

187 The algal growth inhibition tests with *Desmodesmus subspicatus* were following OECD 201
188 guideline (2011) with minor modifications. The algae growth inhibition test was carried out in
189 24-well microplates with a final assay volume of 2 ml per well (Eisentraeger et al. 2004). CIP,
190 CP mixture, NOR, NP mixture, OFL and OP mixture were diluted by the incubation medium in a

1:2 dilution series with three internal replicates: 0.625-20 mg/L, 0.625-20 mg/L, 0.625-10 mg/L, 0.625-10 mg/L, 2.5-80 mg/L, and 0.625-20 mg/L, respectively. Two controls were used, one with 0.1% DMSO and one with millipore water (Millipore, Schwalbach, Germany). The algal biomass was determined at 24, 48 and 72h by relating measured optical densities ($\lambda = 595$ nm) (Infinite 200, Tecan, Crailsheim, Germany). The algal growth inhibition assay was performed in triplicate per concentration.

Statistical analyses were performed using the software package ToxRat Professional XT, version 2.10 (ToxRat® Solutions GmbH, Alsdorf, Germany) based on the logarithmic increase of biomass during the test period. Dose–response curves were plotted by the software Graphpad (Prism 6.0, La Jolla, USA) using log (agonist) vs. response with a variable slope, where the top and bottom of the curve was respectively set to 0% and 100%, respectively. The significance was $p < 0.05$. Evaluations were based on nominal concentrations of FQs and the oxidation mixtures, respectively.

3. Results and discussion

3.1 Electrochemical oxidation of FQs

3.1.1 Identification of transformation products

The voltage – mass intensity curves of the three FQs (Fig.2) were first recorded to determine the reaction starting voltage, while a potential ramp from 0 to 3000 mV with a slope of 10mV/s was applied. The starting voltages of electrochemical oxidation of CIP, NOR and OFL in the EC cell were 1150, 1200 and 1100 mV, respectively.

The mass spectra of the three compounds at constant voltages of 1500, 2000, 2500 and 3000 mV were then recorded to observe the possible oxidation product peaks. Eight possible ciprofloxacin oxidation products (CPs) with masses of 263, 291, 306, 334, 348, 350, 360, and 362 were detected by EC-MS. The electrochemical oxidation of NOR also led to form eight possible products (NPs) with masses of 251, 279, 294, 322, 336, 348, 350, and 368. Furthermore, we observed 8 possible ofloxacin oxidation products (OPs) with masses of 279, 307, 336, 346, 348, 350, 364, 376, and 392. The structural information of the oxidation products was identified by EC coupled to high resolution FTICR-MS. The elemental composition/molecular formulas were created on the basis of the exact masses (error < 2 ppm) with FTICR-MS. The low errors indicated the high grade of confidence in the assignation of the elemental composition. Some products were not detected in the FTICR-MS, possibly due to the differences in sensitivities of mass spectrometry. On the basis of the mass spectrometry analysis data of QTRAP and FTICR-MS, we elucidated the chemical structures of oxidation products (Table 1).

3.1.2 Oxidative mechanism

The voltage – mass intensity curves of each oxidation product were also recorded to observe the transformation trends of these products (Fig.2). We found CP 306, CP 348, CP 350 and CP 362 formed at the same time with the decline of the mass intensity of CIP, and then decreased soon at relatively low oxidation voltage. The results reveal that CIP can be easily and rapidly oxidized to the four products, which will then mostly be oxidized to other products or eliminated with the increase of voltage. CP 334 and CP 360 formed after the first four products, and slightly decreased with the increase of voltage, implying that they were generated at higher oxidation voltages and partly transformed to other products. The products formed at last were CP 263 and CP 291, of which mass intensity increased to the end, indicating that the two products are further oxidation products. We observed similar transformation trends for NPs and OPs (Fig.2).

Based on the structural information and transformation trends of identified oxidation products as well as a literature review, the primary oxidation pathways of the three FQs were proposed (Fig.3). We found that oxidation and cleavage of the piperazinyl ring represent the main oxidation pathways of FQs in our study, while the fluoroquinolone core remained unchanged. Compared to the literature, we believe the piperazinyl ring was first attacked via the hydroxyl radicals generated by the electrode, and then oxidized following the different pathways as shown in Fig.3 (a, b and c).

Pathway I: N-dealkylation processes (Liu et al., 2012 and Thabaj et al., 2007) occurred at the N1 and N4 position on the piperazinyl ring, forming desethylene products (CP 306, NP 294, OP 336). The N1 and N4 positions were attacked by hydroxyl radicals and the oxidation of the alkyl group adjacent to the nitrogen formed unstable alcohol intermediates, which split away, taking an ethylene group with them. Subsequently, CP 306, NP 294 and OP 336 were fully dealkylated to the transformation products CP 263, NP 251 and OP 279, which were also identified in other

oxidation processes (Hubicka et al., 2013 and Ji et al., 2014). In the case of OFL, the N-dealkylation reaction at N4 position also resulted in OP 348, which later was transformed into OP 350.

Pathway II: The hydroxylation of the piperazinyl ring and a subsequent loss of 2H produced a keto-derivative of the piperazinyl ring. Further hydroxylation of the keto-derivative led to hydroxyl-keto-derivative products: CP 362, NP 350 and OP 392, which have been reported previously (Dewitte et al., 2008, Liu et al., 2012 and Zhou and Jiang, 2015). The subsequent loss of the CO group resulted in the opening of the piperaziyl ring, and formed CP 334, NP 322 and OP 364. CP 334 and NP 322 were then dealkylated to CP 291 and NP 279, respectively. OP 364 was first dealkylated to OP 350, which later was transformed into OP 307.

In addition to the products produced by Pathway I and II, several by-products were identified. Amidation of CIP, occurring at N4 position, led to CP 360. The trihydroxylation product of NOR was also detected.

We observed some differences in the generation of desethylene products (CP 306, NP 294, OP 336) proposed above from some other literature (Chen and Chu, 2015, Ji et al., 2014 and Zhang et al., 2015) which proposed their formation from CP 334, NP 322 and OP 364 by loss of CO. As we observed from the voltage – mass intensity dependence curves of oxidation products (Fig. 2), desethylene products formed prior to CP 334, NP 322 and OP 364 at lower voltages, which indicated the desethylene products forms rapidly from parent compounds via N-dealkylation processes during electrochemical oxidation.

Furthermore, the release of inorganic ions was found to be an indicator of the oxidation mechanism (Lin et al., 2013a and 2013b). In our study, the release of F^- in the reaction solutions

was investigated, however, only 4.3%, 5.4% and 7.9% of F^- were found to be released in the reaction solutions of CIP, NOR and OFL, respectively. The results indicate the oxidation at fluoroquinolone core and further mineralization of the parent compounds may only be side reactions due to our reaction conditions, which are consistent with the oxidation mechanisms that we proposed.

Fluoroquinolones are structurally related antibacterial agents that function primarily by inhibiting the DNA-gyrase or topoisomerase activity of bacteria (Shen et al., 1989). Due to the intact fluroquinolone core after electrochemical oxidation, oxidation products of FQs may retain antibacterial activity and potentially result in antibiotic resistance. Furthermore, we hypothesize that the oxidation products still retain their toxicity to aquatic organisms. Therefore, antibacterial activity testing and toxicological evaluation of three FQs and their oxidation products is essential.

3.2 Toxicological evaluation of FQs and their oxidation mixtures

3.2.1 Chemical analysis of scaling up electrochemical oxidation reactions

The oxidation mixtures for ecotoxicological evaluation were prepared by scaling up reactions in "SynthesisCell". The oxidation mixtures were then purified and concentrated by SPE, dried using speedvac, and finally reconstituted into stock solutions. The quantification of the three parent compounds in the stock solutions was conducted by HPLC-MS/MS. The percentages of CIP, NOR and OFL were 7.5% (g/g), 15.5% (g/g) and 2.3% (g/g) respectively, only contributing a minor amount to the mixtures. Although the quantity of primary products cannot be calculated, the relative percentages of parent compounds and their primary oxidation products in the mixtures were calculated based on the UV spectrum (Fig.4) by HPLC-UV/vis (Agilent Technologies, Waldbronn, Germany), assuming the same adsorption factor for all compounds.

The percentages of the parent compounds in the three final oxidation mixtures were lower than the amount of their primary oxidation products.

3.2.2 Residual antibacterial activity

The diameters of inhibition zones of the six samples at different concentrations for *S. typhimurium* are shown in Fig.5. In parallel, the negative controls (Millipore water) and solvent controls (Millipore water with 1% DMSO) were tested and no inhibition zone was observed in either case.

As shown in Fig.5, the zone diameters of the six samples, which reflect the antibacterial activity, decreased with the decrease of exposure concentrations. Comparing the parent compounds to their oxidation mixtures, at the same exposure concentration, CIP, NOR and OFL all formed larger inhibition zones than their oxidation mixtures. This indicates that the oxidation mixtures have a lower antibacterial activity than their parent compounds at the same concentration. Among all the samples of the oxidation mixtures, only the CP mixture (exposure concentration 50 mg/L) showed a significant antibacterial activity with $\geq$ 22 mm zone diameter (EUCAST, 2016), while the parent compounds that CIP (50, 25 and 10 mg/L), NOR (50 and 25 mg/L) and OFL (50 and 25 mg/L) all formed $\geq$ 22 mm inhibition zones. At the lowest concentration (2.5 mg/L), CP mixture, NP mixture and OP mixture showed no antibacterial activity, while CIP, NOR and OFL still formed 17.3, 10.8 and 11.7 mm inhibition zones, respectively. In addition, Fig.5 (c) shows there was already no inhibition zone of the OP mixture observed when its concentration was lower than 50 mg/L. These findings imply that the antibacterial activity apparently declined after electrochemical oxidation, particularly for OFL.

As mentioned previously, quinolone core is believed to be responsible for the antibacterial property of FQs. However, we found although all the identified oxidation products appear to retain the fluoroquinolone core, the antibacterial activity of the oxidation mixtures declined after electrochemical oxidation. Therefore, we hypothesize that the hydroxylation and ring cleavage of piperazine with the fluoroquinolone core intact can also reduce the activity of the three FQs. In a previous study, Paul et al. (2010) observed the decrease of the antibacterial activity in CIP reaction solutions during photo(cata)lytic processes. They further discussed that piperazine transformation may reduce FQ binding affinity to DNA topoisomerase, and consequently diminish the antibacterial potency, which agrees well with our findings.

Nevertheless, the mixtures after electrochemical oxidation still showed measurable antibacterial activity in the microbial tests. To investigate the contribution of the parent compounds in the oxidation mixtures for residual antibacterial activity, we calculated the concentrations of the parent compounds in their mixtures, based on the quantification data by HPLC-MS/MS. The CP mixture with a concentration of 50 mg/L forming 23.8 mm inhibition zone showed similar antibacterial activity to the sample of CIP with 10 mg/L forming 23.3 mm inhibition zone. However, the calculated concentration of CIP in the mixture was only 3.8 mg/L, which implies that CIP only contributes to a part of the activity of the mixture. Similarly, the NP mixture at 50 mg/L, containing 7.8 mg/L NOR, formed 20 mm inhibition zone, which is larger than that of NOR at 10 mg/L (19 mm). The OP mixture (50 mg/L) including 1.15 mg/L OFL formed 11.8 mm inhibition zone, which is equal to OFL at 2.5 mg/L (11.7 mm). These findings indicate that not only the parent compounds in the mixtures caused residual antibacterial activity. In the study of Čvančarová et al. (2015), the responsibility of the products for the residual antibacterial activity after biotransformation was evaluated by Principal Component Analysis

(PCA). They found most of CIP and NOR products still contributed to residual antibacterial activity, while the formation of OFL products resulted in a decrease of the activity for five different strains, which is consistent with our experimental results. Therefore, we assume that the oxidation products of the three FQs retained the antibacterial property, which may also contribute to residual antibacterial activity.

Although the antibacterial activity of the FQs was reduced after electrochemical oxidation, the residual activity of the oxidation mixtures still should be considered.

3.2.3 Toxicity change on green algae

In order to investigate the toxicity change of the FQs during electrochemical oxidation, a growth inhibition test (*D. subspicatus*) was used to evaluate the toxicity of the FQs and their oxidation mixtures.

Concentration – response curves of the algal growth inhibition test were shown in Fig.6. The EC_{50} values of the parent compounds and their oxidation mixtures for the end point of the growth rate, after 72 h exposure, were calculated by ToxRat software. The EC_{50} values are first obtained on the specie *D. subspicatus*. The EC_{50} values of the CP mixture (8.7 mg/L) and the NP mixture (6.7 mg/L) was found to be almost the same as that of their parent compounds (CIP: 8.8 mg/L, NOR: 6.8 mg/L). Whereas the EC_{50} values of the OP mixture (26.8 mg/L) are much lower than that of OFL (102.7 mg/L) after 72h exposure. Our results showed that the toxicity of the oxidation mixtures is equivalent or even higher than that of their parent compounds. This observation indicates the algal toxicity of the FQs increased after electrochemical oxidation.

Photoautotrophic microalgae, as primary producers that supply nutrients for the rest of the aquatic biota, play a crucial role in the structure of the whole aquatic ecosystem. Therefore,

358 microalgae are often considered as a good indicator for xenobiotics and water quality. Table 2
359 summarizes the EC_{50} values of the FQs obtained in this study and the toxicity data reported in
360 the literature for the three FQs on different green algal species. Although there is no toxicity data
361 of the oxidation mixtures on green algae available in previous works, the toxicity increase after
362 electrochemical oxidation observed in the present study emphasizes the potential risks of the
363 oxidation mixtures in the aquatic environment.

364 Several studies on toxicity change of FQs during other oxidation processes showed different
365 results. De Bel et al. (2009) investigated the toxicity change of CIP on *P. subcapitata* at different
366 pH values, and observed a dramatic increase of growth inhibition after 20 min of ultrasonic
367 irradiation. Vasquez et al. (2013) observed genotoxicity in the photo(cata)lytically treated
368 solutions of OFL, however, the effects decreased to non-toxic level after continuously increasing
369 irradiation time. In the study of Michael et al. (2010), different toxicity profiles for treated
370 effluents of OFL by the solar Fenton and TiO_2 methods on *D. magna* were observed. The
371 toxicity of the solution treated by solar Fenton processes dramatically increased at 30 and 60 min
372 treatment time, while the toxicity only slightly increased using TiO_2 photocatalysis. In Carbajo et
373 al. (2015) study, the toxicity of OFL decreased in four bioassays with an increasing ozone
374 dosage during ozonation treatment. We assume that the toxicity change of FQs differs in
375 different oxidation processes, and likely depends on oxidation mechanisms, reaction time, and
376 oxidative strength. However, the formation of toxic intermediates during oxidation processes
377 was confirmed by most investigations and is in good agreement with the results of our study.

Conclusions

The purpose of the current study was to understand oxidation mechanisms of FQs during electrochemical oxidation processes and evaluate the antibacterial activities and algal toxicity of FQs and their oxidation products.

- Electrochemical oxidation products of the three FQs were identified and oxidation pathways were proposed. Electrochemical oxidation mechanisms of FQs on a BDD electrode were hydroxylation and cleavage of piperizinyl ring via hydroxyl radicals. Our study shows a general understanding of the FQs transformation during electrochemical oxidation processes, which is indispensible for the optimization of the treatment of wastewater containing FQs.
- Most of CPs and NPs retained antibacterial activities, although at a lower level than that of their parent compounds. The antibacterial activity of OFL had an appreciable decrease after electrochemical oxidation. Although the antibacterial activity of FQs reduced after EAOPs, we should not ignore the potential risk of FQs and their oxidation products inducing drug resistance, especially in hospital effluents containing high concentrations of FQs.
- The mixtures of FQs after electrochemical oxidation showed equivalent and even higher toxicity to green algae (*D. subspicatus*) compared to their parent compounds. The results indicate that the oxidation of the piperizinyl ring cannot remove the toxicity of the FQs and may generate toxic oxidation products.

In summary, we found that the piperazinyl ring of FQs was completely oxidized while the fluoroquinolone ring remained intact during electrochemical oxidation on a BDD electrode. Due to the residual antibacterial activity and algal toxicity increase observed in the present study, the oxidation of the piperizinyl ring is not sufficient to eliminate the toxicological effects of FQs.

Stronger electrochemical oxidation of FQs by introducing strong oxidative agents or in combination with other technologies should be studied in further investigations.

Acknowledgements

The first author is grateful for the support by China scholarship Council and Helmholtz Association of German Research Centers. The first author is grateful for the help by Nicole Asante on the revision of the paper. The authors thank Project SIGN funded by Client-Programs (02WCL1336).

References:

Bengtsson-Palme, J. and Larsson, D.G.J., 2015. Antibiotic resistance genes in the environment: prioritizing risks. Nat Rev Micro 13 (6), 396-396.

Bos, J., Zhang, Q., Vyawahare, S., Rogers, E., Rosenberg, S. M. and Austin, R. H., 2015. Emergence of antibiotic resistance from multinucleated bacterial filaments. Proceedings of the National Academy of Sciences of the United States of America 112 (1), 178-183.

Carbajo, J. B., Petre, A. L., Rosal, R., Herrera, S., Letón, P., García-Calvo, E., Fernández-Alba, A. R. and Perdigón-Melón, J. A., 2015. Continuous ozonation treatment of ofloxacin: Transformation products, water matrix effect and aquatic toxicity. Journal of Hazardous Materials 292, 34-43.

Carlesi Jara, C., Fino, D., Specchia, V., Saracco, G. and Spinelli, P., 2007. Electrochemical removal of antibiotics from wastewaters. Applied Catalysis B: Environmental 70 (1-4), 479-487.

Chen, L., Hofmann, D., Klumpp, E., Xiang, X., Chen, Y. and Küppers, S., 2012. Bottom-up approach for the reaction of xenobiotics and their metabolites with model substances for natural organic matter by electrochemistry–mass spectrometry (EC-MS). Chemosphere 89 (11), 1376-1383.

Chen, L., Kuppers, S., Wang, Z., Xiang, X. and Cao, S., 2014. Online electro-Fenton-mass spectrometry reveals 2,4',5-trichlorobiphenyl oxidation products and binding to organic matter. Environmental Chemistry Letters 12 (2), 329-334.

Chen, M. and Chu, W., 2015. Photocatalytic degradation and decomposition mechanism of fluoroquinolones norfloxacin over bismuth tungstate: Experiment and mathematic model. Applied Catalysis B-Environmental 168, 175-182.

Čvančarová, M., Moeder, M., Filipová, A. and Cajthaml, T., 2015. Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi - Metabolites, enzymes and residual antibacterial activity. Chemosphere 136, 311-320.

De Bel, E., Dewulf, J., Witte, B. D., Van Langenhove, H. and Janssen, C., 2009. Influence of pH on the sonolysis of ciprofloxacin: Biodegradability, ecotoxicity and antibiotic activity of its degradation products. Chemosphere 77(2), 291-295.

Dewitte, B., Dewulf, J., Demeestere, K., De Vyvere, V. V., De Wispelaere, P. and Van Langenhove, H., 2008. Ozonati ciprofloxacin on of in water: HRMS identification of reaction products and pathways. Environmental Science & Technology 42 (13), 4889-4895.

Ebert, I., Bachmann, J., Kuhnen, U., Kuster, A., Kussatz, C., Maletzki, D. and Schluter, C., 2011. Toxicity of the Fluoroquinolone Antibiotics Enrofloxacin and Ciprofloxacin to Photoautotrophic Aquatic Organisms. Environmental Toxicology and Chemistry 30 (12), 2786-2792.

Eguchi, K., Nagase, H., Ozawa, M., Endoh, Y. S., Goto, K., Hirata, K., Miyamoto, K. and Yoshimura, H., 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57 (11), 1733-1738.

Eisentraeger, A., Brinkmann, C., Michel, K., Hahn, S., Huettner, M. and Weber, G., 2004. Development of automated high-throughput ecotoxicity and genotoxicity test systems and fields of application. Water Science and Technology 50 (5), 109-114.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxēaus, N., Giudice, R. L., Pollio, A. and Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: Are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? Environmental Toxicology and Chemistry 23(5), 1344-1354.

González-Pleiter, M., Gonzalo, S., Rodea-Palomares, I., Leganés, F., Rosal, R., Boltes, K., Marco, E. and Fernández-Piñas, F., 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: Implications for environmental risk assessment. Water Research 47 (6), 2050-2064.

Guinea, E., Brillas, E., Centellas, F., Cañizares, P., Rodrigo, M. A. and Sáez, C., 2009. Oxidation of enrofloxacin with conductive-diamond electrochemical oxidation, ozonation and Fenton oxidation. A comparison. Water Research 43 (8), 2131-2138.

456 Guinea, E., Garrido, J.A., Rodríguez, R. M., Cabot, P. L., Arias, C., Centellas, F. and Brillas, E., 2010.
457 Degradation of the fluoroquinolone enrofloxacin by electrochemical advanced oxidation processes
458 based on hydrogen peroxide electrogeneration. Electrochimica Acta 55 (6), 2101-2115.
459 Halling-Sorensen, B., Lutzhoft, H.-C. H., Andersen, H. R. and Ingerslev, F., 2000. Environmental risk
460 assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. Journal of
461 Antimicrobial Chemotherapy 46, 53-58.
462 Hambitzer, G. and Heitbaum, J., 1986. Electrochemical thermospray mass spectrometry. Analytical
463 Chemistry 58(6), 1067-1070.
464 Hoffmann, T., Hofmann, D., Klumpp, E. and Küppers, S., 2011. Electrochemistry-mass spectrometry for
465 mechanistic studies and simulation of oxidation processes in the environment. Analytical and
466 Bioanalytical Chemistry 399 (5), 1859-1868.
467 Hubicka, U., Żmudzki, P., Żuromska-Witek, B., Zajdel, P., Pawłowski, M. and Krzek, J., 2013.
468 Separation and characterization of ciprofloxacin, difloxacin, lomefloxacin, norfloxacin, and ofloxacin
469 oxidation products under potassium permanganate treatment in acidic medium by UPLC-MS/MS.
470 Talanta 109, 91-100.
471 Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L. and Parrella, A., 2005. Toxic and genotoxic
472 evaluation of six antibiotics on non-target organisms. Science of The Total Environment 346 (1–3),
473 87-98.
474 Ji, Y., Ferronato, C., Salvador, A., Yang, X. and Chovelon, J. M., 2014. Degradation of ciprofloxacin and
475 sulfamethoxazole by ferrous-activated persulfate: Implications for remediation of groundwater
476 contaminated by antibiotics. Science of The Total Environment 472, 800-808.
477 Johansson, C. H., Janmar, L. and Backhaus, T., 2014. Toxicity of ciprofloxacin and sulfamethoxazole to
478 marine periphytic algae and bacteria. Aquatic Toxicology 156, 248-258.
479 Karst, U., 2004. Electrochemistry/Mass Spectrometry (EC/MS) - A New Tool To Study Drug Metabolism
480 and Reaction Mechanisms. Angewandte Chemie International Edition 43 (19), 2476-2478.

481 Kostich, M. S., Batt, A. L. and Lazorchak, J. M., 2014. Concentrations of prioritized pharmaceuticals in
482 effluents from 50 large wastewater treatment plants in the US and implications for risk estimation.
483 Environmental Pollution 184, 354-359.
484 Kümmerer, K., 2009. Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75 (4),
485 417-434.
486 Kümmerer, K., 2009. Antibiotics in the aquatic environment - A review - Part II. Chemosphere 75 (4),
487 435-441.
488 Lin, H., et al., 2013a. Electrochemical mineralization of sulfamethoxazole by Ti/SnO2-Sb/Ce-PbO2
489 anode: Kinetics, reaction pathways, and energy cost evolution. Electrochimica Acta 97, 167-174.
490 Lin, H., et al., 2013b. Highly Efficient and Mild Electrochemical Mineralization of Long-Chain
491 Perfluorocarboxylic Acids (C9–C10) by Ti/SnO2–Sb–Ce, Ti/SnO2–Sb/Ce–PbO2, and Ti/BDD
492 Electrodes. Environmental Science & Technology 47(22), 13039-13046.
493 Liu, C., Nanaboina, V., Korshin, G. V. and Jiang, W., 2012. Spectroscopic study of degradation products
494 of ciprofloxacin, norfloxacin and lomefloxacin formed in ozonated wastewater. Water Research 46
495 (16), 5235-5246.
496 Magdaleno, A., Saenz, M. E., Juárez, A. B. and Moretton, J., 2015. Effects of six antibiotics and their
497 binary mixtures on growth of Pseudokirchneriella subcapitata. Ecotoxicology and Environmental
498 Safety 113, 72-78.
499 Marselli, B., Garcia-Gomez, J., Michaud, P. A., Rodrigo, M. A. and Comninellis, C., 2003.
500 Electrogeneration of hydroxyl radicals on boron-doped diamond electrodes. Journal of the
501 Electrochemical Society 150 (3), D79-D83.
502 Martins, N., Pereira, R., Abrantes, N., Pereira, J., Gonçalves, F. and Marques, C. R., 2012.
503 Ecotoxicological effects of ciprofloxacin on freshwater species: data integration and derivation of
504 toxicity thresholds for risk assessment. Ecotoxicology 21 (4), 1167-1176.

505 Matuschek, E., Brown, D. F. J. and Kahlmeter, G., 2014. Development of the EUCAST disk diffusion
506 antimicrobial susceptibility testing method and its implementation in routine microbiology
507 laboratories. Clinical Microbiology and Infection 20 (4), O255-O266.
508 Michael, I., Hapeshi, E., Michael, C. and Fatta-Kassinos, D., 2010. Solar Fenton and solar TiO2 catalytic
509 treatment of ofloxacin in secondary treated effluents: Evaluation of operational and kinetic
510 parameters. Water Research 44 (18), 5450-5462.
511 Moreira, F. C., Garcia-Segura, S., Boaventura, R. A. R., Brillas, E. and Vilar, V. J. P., 2014. Degradation
512 of the antibiotic trimethoprim by electrochemical advanced oxidation processes using a carbon-PTFE
513 air-diffusion cathode and a boron-doped diamond or platinum anode. Applied Catalysis B-
514 Environmental 160, 492-505.
515 Nie, X., Wang, X., Chen, J., Zitko, V. and An, T., 2008. Response of the freshwater alga Chlorella
516 vulgaris to trichloroisocyanuric acid and ciprofloxacin. Environmental Toxicology and Chemistry 27
517 (1), 168-173.
518 OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD
519 Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.
520 Paul, T., Dodd, M. C. and Strathmann, T. J., 2010. Photolytic and photocatalytic decomposition of
521 aqueous ciprofloxacin: Transformation products and residual antibacterial activity. Water Research
522 44 (10), 3121-3132.
523 Robinson, A. A., Belden, J. B. and Lydy, M. J., 2005. Toxicity of fluoroquinolone antibiotics to aquatic
524 organisms. Environmental Toxicology and Chemistry 24 (2), 423-430.
525 Shen, L. L., Mitscher, L. A., Sharma, P. N., O'Donnell, T. J., Chu, D. W. T., Cooper, C. S., Rosen, T. and
526 Pernet, A. G., 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: a
527 cooperative drug-DNA binding model. Biochemistry 28 (9), 3886-3894.
528 Sirés, I. and Brillas, E., 2012. Remediation of water pollution caused by pharmaceutical residues based on
529 electrochemical separation and degradation technologies: A review. Environment International 40,
530 212-229.

Thabaj, K. A., Kulkarni, S. D., Chimatadar, S. A. and Nandibewoor, S. T., 2007. Oxidative transformation of ciprofloxacin by alkaline permanganate - A kinetic and mechanistic study. Polyhedron 26 (17), 4877-4885.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2016. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0. http://www.eucast.org.

Van Doorslaer, X., Dewulf, J., Van Langenhove, H. and Demeestere, K., 2014. Fluoroquinolone antibiotics: An emerging class of environmental micropollutants. Science of The Total Environment 500–501, 250-269.

Vasquez, M. I., Garcia-Kaufer, M., Hapeshi, E., Menz, J., Kostarelos, K., Fatta-Kassinos, D. and Kummerer, K., 2013. Chronic ecotoxic effects to Pseudomonas putida and Vibrio fischeri, and cytostatic and genotoxic effects to the hepatoma cell line (HepG2) of ofloxacin photo(cata)lytically treated solutions. Science of The Total Environment 450, 356-365.

Volk, K. J., Yost, R. A. and Brajter-Toth, A., 1989. On-line electrochemistry/thermospray/tandem mass spectrometry as a new approach to the study of redox reactions: the oxidation of uric acid. Analytical Chemistry 61(15), 1709-1717.

Yahya, M. S., Oturan, N., El Kacemi, K., El Karbane, M., Aravindakumar, C. T. and Oturan, M. A., 2014. Oxidative degradation study on antimicrobial agent ciprofloxacin by electro-fenton process: Kinetics and oxidation products. Chemosphere 117, 447-454.

Yang, L.-H., Ying, G.-G., Su, H.-C., Stauber, J.L., Adams, M.S. and Binet, M.T., 2008. Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga Pseudokirchneriella subcapitata. Environmental Toxicology and Chemistry 27 (5), 1201-1208.

Zhang, Q., Jia, A., Wan, Y., Liu, H., Wang, K., Peng, H., Dong, Z. and Hu, J., 2014. Occurrences of Three Classes of Antibiotics in a Natural River Basin: Association with Antibiotic-Resistant Escherichia coli. Environmental Science & Technology 48 (24), 14317-14325.

555 Zhang, X., Li, R., Jia, M., Wang, S., Huang, Y. and Chen, C., 2015. Degradation of ciprofloxacin in
556 aqueous bismuth oxybromide (BiOBr) suspensions under visible light irradiation: A direct hole
557 oxidation pathway. Chemical Engineering Journal 274, 290-297.
558 Zhou, Z. and Jiang, J.-Q., 2015. Reaction kinetics and oxidation products formation in the degradation of
559 ciprofloxacin and ibuprofen by ferrate(VI). Chemosphere 119, S95-S100.
560 Zhu, L., Santiago-Schubel, B., Xiao, H., Thiele, B., Zhu, Z., Qiu, Y., Hollert, H. and Kuppers, S., 2015.
561 An efficient laboratory workflow for environmental risk assessment of organic chemicals.
562 Chemosphere 131, 34-40.