Title: Fate of enrofloxacin in lake sediment: Biodegradation, transformation product identification, and ecotoxicological implications

Authors: Tanare C. R. Ferreira[1]; Maranda Esterhuizen-Londt[2,3*]; Marcelo Zaiat[1]; Stephan Pflugmacher[2,3]

[1] Laboratory of Biological Processes, Centre for Research, Development and Innovation in Environmental Engineering, São Carlos School of Engineering, University of São Paulo (USP), 1100 João Dagnone Ave., Santa Angelina, Zip Code: 13563-120, São Carlos, São Paulo, Brazil

[2] University of Helsinki, Faculty of Biological and Environmental Sciences, Ecosystems and Environment Research Programme, Aquatic Ecotoxicology in an Urban Environment, Niemenkatu 73, 15140 Lahti, Finland

[3] Korea Institute of Science and Technology Europe (KIST), Joint Laboratory of Applied Ecotoxicology, Campus 7.1, 66123 Saarbrücken, Germany

Dr. Tanare C. R. Ferreira: Email: tanarecambraia@gmail.com

Dr. Maranda Esterhuizen-Londt (* Corresponding author): Orcid: https://orcid.org/0000-0002-2342-3941), Email: maranda.esterhuizen-londt@helsinki.fi, Phone: +358 503188337

Prof. Dr. Marcelo Zaiat: Email: zaiat@sc.usp.br

Prof. Dr. Stephan Pflugmacher: Orcid: https://orcid.org/0000-0003-1052-2905, Email: stephan.pflugmacher@helsinki.fi

Declaration of interest: none

Abstract

Various pharmaceutical drugs are being detected in different environmental compartments such as surface waters, groundwater, and sediment; a major concern since they are biologically active substances which can interfere with biological systems affecting the native biota. Among these drugs, antimicrobials are especially worrisome mainly due to the development of bacterial resistance. The aims of the present study were to investigate if enrofloxacin, an emergent antibiotic pollutant, could be biodegraded in lake sediment, identify its break down products and to determine if these products have antimicrobial properties or are toxic. Three biodegradation products were identified and the antibiotic susceptibility assay proved that the products formed did not display antibiotic effects. Ecotoxicity testing with green algae suggested that the degradation products do not cause adverse effects statistically. However, it is suggested that further investigations are needed to identify the mechanism of degradation and the microbes involved.

Keywords: Enrofloxacin, biodegradation, lake sediment, antibiotic susceptibility, ecotoxicology

1. Introduction

The presence and persistence of pharmaceuticals in the environment and their fate are increasingly pertinent issues (Álvarez-Ruiz et al., 2015; Ngumba et al., 2016). Parallel to this, there are concerns regarding the preservation of aquatic ecosystems and the potential contamination risk of public water supplies. This has encouraged studies aimed at identifying and quantifying pharmaceutical waste in the environment so that the risk posed can be evaluated, and subsequently, the disposal thereof can be minimized and efficient processes to remove these drugs can be developed (Maranho et al., 2014; Mwanamoki et al., 2014; Álvarez-Ruiz et al., 2015).

Considering the significant threat to humans, animals, and agriculture, antibiotics are one of the most relevant emerging pollutants in the environment. Their continuous interactions with and inhibitory effects on microorganisms may cause damage, including antibiotic resistance induction (Adachi et al., 2013). Fluoroquinolones, such as enrofloxacin, are broad-spectrum synthetic antibiotics commonly used in human and veterinary medicine (Trouchon and Lefebvre, 2016) as well as in agriculture and aquaculture (Migliore et al., 1996). Fluoroquinolone antibiotics typically have a fluoro group attached to the central ring structure at position 6. Enrofloxacin is distinguished by three ionisable functional groups, namely a piperazine substituent at N-4, a dihydroquinoline ring at N-1, and the 3-carbonyl group (Fig 1). These antibiotics show strong antibacterial activity and are somewhat resistant to abiotic and biotic degradation (Migliore et al., 1996). Orally administered as medicines, only a small percentage of fluoroquinolones are adsorbed and metabolized, the rest is excreted. For most fluoroquinolones, the elimination half-life has been reported to be 16 h and it is therefore likely that they will be excreted largely unchanged with less than 25% metabolization. Within this context, fluoroquinolones are acknowledged pollutants that have already been detected in different environmental compartments, such as up to 248 ng l^{-1} in surface waters (Wagil et al.,

63 2014), up to 49 ng l^{-1} in groundwater (Ma et al., 2015), and up to 7.7 mg kg^{-1} in sediment (Hu
64 et al., 2012) have been reported.

65 **Figure 1 here.**

66 Another important factor to consider regarding pharmaceutical pollution is that during
67 wastewater treatment or even in the environment, the pollutants may be only partially
68 degraded and thus numerous transformation products (TPs) are generated which may be more
69 toxic than the precursor molecules (Escher and Fenner, 2011). Generally, drugs and their TPs
70 are found in sub-µg l^{-1} concentrations in unknown complex matrices making it necessary to
71 use analytical methods of high sensitivity and selectivity to detect and identify them.
72 Moreover, the lack of analytical standards for TPs complicates the analysis thereof.
73 Identifying unknown compounds where standards are not available is challenging. The first
74 step is assessing whether prediction of TPs using computational (in-silico) prediction tools is
75 possible. Furthermore, a proper prediction of their formation may be done considering the
76 organism or the system where the TPs are formed (Bletsou et al., 2015). In a second step,
77 when it is possible to draw up a list of potential TPs assembled from the literature or from
78 prediction models, a suspect screening can be done in samples for those candidates. However,
79 whenever predictions are unavailable, non-target screening analyses are performed to identify
80 novel TPs with sophisticated post-acquisition data tools, like MZmine
81 (http://mzmine.sourceforge.net/), and supplementary analytical techniques (Bletsou et al.,
82 2015).

83 The abiotic and biotic degradation of several pharmaceuticals in sediments and the water
84 column are well understood (Löffler et al., 2005; Jiang et al., 2010), however, little data on
85 the fate of enrofloxacin are available and if its TPs are equally, or potentially even more
86 harmful than the parent compound. Due to the inability of wastewater treatment processes to

fully eliminate pharmaceuticals, together with various contamination input sources, antibiotics such as fluoroquinolones, especially enrofloxacin, are bound to end up in the environment as already evident from monitoring studies (reviewed by Homem and Santos, 2011). It is therefore important to understand the environmental fate of these compounds and the ecological threat their natural breakdown products may pose to the environmental compartments they accumulate in.

The aims of the present study were, therefore, to identify the TPs of enrofloxacin in lake sediments and to test the formed TPs for antimicrobial properties and their ecotoxicological effects using a modified antimicrobial susceptibility test and a green algae growth test, respectively, to understand the fate and effects of enrofloxacin pollution on the environment, specifically freshwater lakes..

2. Materials and methods

2.1 Biodegradation with lake sediments

Sediment samples were collected from Müggelsee, a lake in the eastern suburbs of Berlin, the capital city of Germany, approximately 200 m from the shoreline by removing the first 15 cm of sediment using a bottom sampler. After drying the sediment at 30°C, a dry weight of 1 g per sediment sample was used per replicate for both the treatment and the controls. Three controls were prepared, i.e. 1) 10 mg l^{-1} enrofloxacin in 10 ml water to test its natural degradation, 2) 10 ml water lacking enrofloxacin in the natural sediment from Müggelsee to investigate if the sediment was previously contaminated with enrofloxacin, and 3) 10 mg l^{-1} enrofloxacin (10 ml) in sterilized sediment to eliminate the influence of the native microflora. The sediment samples were sterilized by autoclaving at 121°C for 35 min (16 psi). For the treatment samples, 10 ml of 10 mg enrofloxacin l^{-1} was added to the natural sediments samples from Müggelsee. The samples were incubated at 20°C, shaking at 145 rpm, in the absence of light. Thereafter, batch experiments were performed in duplicate for different periods of time exposures (5, 24, 48, and 72 h). After centrifugal separating at 1700 × g, 1 ml of supernatant was collected for direct analysis, after filtration. To concentrate the remaining supernatant volume, the samples were lyophilized and reconstituted with 1 ml of a 5% acetonitrile solution in ultrapure water. The sediments from the treatment samples were extracted with sequential acetonitrile and methanol solvents steps. The extracts were lyophilized and reconstituted in 1 ml of a 5% acetonitrile solution in ultrapure water and analysed. The recovery of enrofloxacin after concentration was test in methanol, acetonitrile, and water (n=3) by spiking with 1 mg l^{-1} enrofloxacin before freeze drying at −50.3 °C and a pressure of 6.1 mbar in a Lio 5P lyophilisator (Kambič Laboratorijska oprema). The method recovery percentage ranged from 76 to 103% for the three solvents.

The exposure concentration of 10 mg l^{-1} enrofloxacin (soluble up to 146 mg l^{-1} in water) was selected in order for all breakdown products produced to be identified, i.e. that the concentration of a specific TP did not fall below the limit of detection (10 pg on column) and quantification (50 pg on column). Also, the concentration selected also serves as a worst case scenario to assess the effect of breakdown products of enrofloxacin in the environment.

2.2 Product identification using qualitative analysis

The prepared samples were analysed using Liquid Chromatography Electrospray Ionization Quadrupole Ion-Mobility Time-of-Flight Mass Spectrometry (LC-ESI-IMS-TOF) (Waters Co.), subjected to high-resolution mass spectrometry. Chromatographic separation was achieved on a Kinetex C18 column (100 mm x 2.1 mm; 2.6 µm; Phenomenex) eluted with mixtures of 0.1% formic acid in ultrapure water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) starting with 5% B for 3 min. Over the next 9 min, mobile phase B was increased from 5 to 60% and further to 95% over the next 2 min. From the 14^{th} to the 20^{th} min mobile phase B was kept constant at 95% B. Over the next 2 min phase B was reduced to 5% and the column was allowed to re-equilibrate for 4 min before the next injection. The oven temperature was set at 40°C, the flow rate was 0.25 ml min^{-1} and the injection volume was 5 µl. The mass spectrometric analyses were performed in the positive ionization mode (electrospray), and the operating conditions were as follows: drying gas flow: 8 l min^{-1}; desolvation temperature: 200°C; capillary voltage: 4.5 kV; nebulizer pressure: 4 bar; spectra acquisition rate: 2 Hz at full MS mode, operating with a scan range from m/z 50 to m/z 1000. Using the described analytical settings, enrofloxacin had a retention time of 6.51 min and m/z of 360.1. MZmine 2 (Version 2.21) which is a modular framework for processing, visualizing,

and analysing mass spectrometry-based molecular profile data, was used for data extraction, deconvolution, and alignment (Bletsou et al., 2015).

2.3 Ecotoxicological tests

The freshwater algae growth rate inhibition test with the single-celled green algae *Desmodesmus subspicatus* was performed according to the DIN EN ISO 8692:2012-06.

For the antibiotic susceptibility assay, three controls at the inception of the experiment were prepared, i.e. Provisoli medium (Pflugmacher et al., 2006) as negative control for media samples, enrofloxacin at a concentration of 10 mg l^{-1} in media as a positive control, and unexposed sediment as a negative sediment control. The effect of the sediment microbe population on the antibiotic nature of enrofloxacin was evaluated by preparing a negative control of media and sediment, 10 mg l^{-1} enrofloxacin in media together with the sediment, and 10 mg l^{-1} enrofloxacin in media with autoclaved sediment. All exposure sets were conducted in quadruplicate.

A modified version of the Kirby Bauer disk diffusion assay (Bauer et al. 1996), was used to evaluate the bactericidal effect of enrofloxacin after treatment with the sediments microbes. In short, single colonies of *Escherichia coli* Top 10 (ThermoFisher Scientific) were aseptically transferred to nutrient broth and cultivated overnight at 37°C. Thereafter, spread plates of the culture were prepared on nutrient agar (prepared according to supplier specifications) and allowed to dry for 5 minutes. Sterile diffusion disks were dipped in each of the samples and placed on the prepared plates (one disk per replicate per plate). The plates were incubated overnight at 37°C. The inhibition zone radius per replicate was determined in millimetre.

2.4 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (version 21, SPSS, Inc., Chicago, IL, USA; $\alpha = 0.05$, 95 % CI). Data were tested for normality and homogeneity of variance using Shapiro-Wilk test and Levene's test, respectively. A one-way analysis of variance test was performed followed by a Turkey´s post-hoc-test to identify significant differences between the treatments and controls ($\alpha = 0.05$).

172 **3. Results and discussion**

173 3.1 Degradation products identification

174 For the first three sampling points, i.e. 5, 24, and 48 h, the concentration of enrofloxacin
175 remained statistically constant in the controls and treatments ($p > 0.05$) and no TP products
176 could be identified, probably as the concentration of the TPs were below the limit of
177 detection. The results obtained in the control experiments are presented in Fig 2. Comparing
178 Fig 2A versus 2B it is evident that during the three days of exposure, the enrofloxacin
179 concentration of 10 mg l^{-1} in media remained unchanged ($p > 0.05$), demonstrating its stability
180 under the experimental conditions. Fig 2C showed that no free enrofloxacin could be detected
181 in the untreated sediment..

182 **Figure 2 here.**

183 In the control experiment, which consisted of enrofloxacin in the sterile sediment, neither
184 enrofloxacin nor TPs could, however, not be detected after 72 h of incubation (Fig 2D)
185 suggesting that it could have adsorbed to the sediment. Fluoroquinolones have previously
186 been reported to form strong bonds with ions such Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+} causing them to
187 adsorb onto sewage, sludge, soil, and sediment, which is said to cause their environmental
188 resilience and resistance to microbial degradation (Al-Ahmad et al., 1999; Ingerslev and
189 Halling-Sørensen, 2000; Kümmerer et al., 2000). Enrofloxacin is said to have a very high
190 affinity for sludge, soils, and sediments (Van Doorslaer et al., 2014). Compared to other
191 antibiotics, fluoroquinolones have a very high sorbent coefficient of 260 to 5610 l kg^{-1}
192 (Nowara et al., 1997). It was previously shown that the adsorption of fluoroquinolones onto
193 clay surfaces is attributed to the carboxylic acid moiety binding the positively charged clay
194 surface (Stern layer), which coincidentally is also the functional moiety responsible for gyrase
195 binding together with the ketone of C4 (Nowara et al., 1997; Marengo et al., 1997).

196 After 72 h, no enrofloxacin could be detected in the treated samples. Using the software
197 MZmine, after peak deconvolution and alignment of the chromatograms obtained from the
198 treatment and control samples, three compounds were identified in the enrofloxacin treated
199 natural sediment samples, i.e. two degradation products were found in the concentrated media
200 samples (m/z 308 and m/z 332) and another in the sediment extracts (m/z 318).

201 **Figure 3 here.**

202 Fig 3 (A to C) presents the high-resolution MS/MS spectrums obtained to confirm the
203 proposed structures. All degradation products identified were formed through modifications
204 occurring in the essential structure of the quinolones. For all three TPs, the piperazine ring
205 remained unchanged (Fig 4). The two TPs in the concentrated media samples were identified
206 as 2-Cyclopropylamino-4-(4-ethyl-1-piperazinyl)-5-fluorobenzoic acid (m/z 308) and 1-
207 Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-3-hydroxy-4-1H-quinolinone (m/z 332), and
208 the TP in the sediment extract was identified as 1-Cyclopropyl-6-(4-ethyl-1-piperazinyl)-5-
209 fluoro-1H-indole-2,3-dione (m/z 318). The degradation of enrofloxacin by the brown rot
210 fungus *Gloeophyllum striatum* and the metabolites formed were previously investigated
211 (Wetzstein et al., 1997). All three the degradation products identified in the present study
212 were also described by Wetzstein et al. (1997), suggesting that the degradation could be
213 attributed to microbial degradation. As no degradation products were detected in the control,
214 where enrofloxacin was incubated with sterile sediment, this hypothesis is further supported,
215 however, this should be further investigated in future to investigate if microbes were involved
216 and if so, which microbes were responsible for the TPs identified.

217 **Figure 4 here.**

218 Decarboxylation occurred in the essential structure of the enrofloxacin (Fig 4), which
219 irreversibly inactivates the drug because the carboxyl group is essential for the antibacterial

220 activity of fluoroquinolones (Domagala, 1994). The cleavage of the heterocyclic core of
221 enrofloxacin was observed in the intermediate A and B (Fig 4).

222 3.2 Ecotoxicological tests

223 The algae growth rate inhibition test (Fig 5) showed that the TPs formed had no significant
224 effect on the specific growth rate of *D. subspicatus* compared to when cultivated in growth
225 media only (negative control) ($p = 0.087$). Interestingly, enrofloxacin was previously reported
226 to be toxic to green algae with an EC_{50} of 5,568 µg l^{-1} (Ebert et al., 2011). Yet, in the present
227 study, a concentration of 10 mg l^{-1} enrofloxacin had no statistical effect on the specific growth
228 rate compared to that of the control ($p = 0.426$).

229 The microalgae displayed the best specific growth rate in the samples from which
230 enrofloxacin was incubated in sterile sediment for three days. It is plausible that the
231 enrofloxacin was bound to the sediment, therefore unable to adversely affect the algae. The
232 algae also could have benefitted from the addition of minerals and micronutrients supplied
233 form samples in contact with the sediment.

234 **Figure 5 here.**

235 The antibiotic susceptibility assay (Fig 6) showed that after the 72 h biodegradation period of
236 enrofloxacin (Treatment (sediment) and (liquid)), the products formed in the solution as well
237 as those bound to the sediment, lost their antibiotic effects compared to the 10 mg l^{-1}
238 enrofloxacin solution (Enro initial) ($p < 0.05$). This was expected as it was shown for the TP
239 identification that enrofloxacin was decarboxylated. Interestingly, incubation of enrofloxacin
240 with the sterilized sediment reduced the antibacterial properties of the enrofloxacin by 1.8-
241 fold in the sediment (Positive control (sediment)) and 3.2-fold in the media (Positive control
242 (media)) ($p < 0.05$). As sediment binding was previously proposed to occur via the carboxyl

243 group (Marengo et al., 1997), which is essential for its antibacterial activity (Wetzstein,

244 2001), complete loss of the property was expected.

245 **Figure 6 here.**

4. Conclusions

The applied strategy for the analysis of the biodegradation of enrofloxacin in lake sediment allowed the detection and the identification of three TPs. All TPs identified were formed through modifications occurring in the essential structure of the quinolones, however, the piperazine ring remained unchanged. The antibiotic susceptibility assay showed that, after biodegradation, the products formed in the solution lost the antibiotic effects. In support, the algal growth inhibition assay suggests that the degradations products formed did not affect the growth rate of algae. It is also suggested that further investigations are needed to obtain an in-depth understanding of the effects of enrofloxacin degradation products on organisms existing in aquatic environments.

5. Acknowledgments

The authors gratefully acknowledge the support provided for this study by the São Paulo Research Foundation (FAPESP) [Grant number 2014/19650-8 and 2015/24186-1].

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Figure captions:

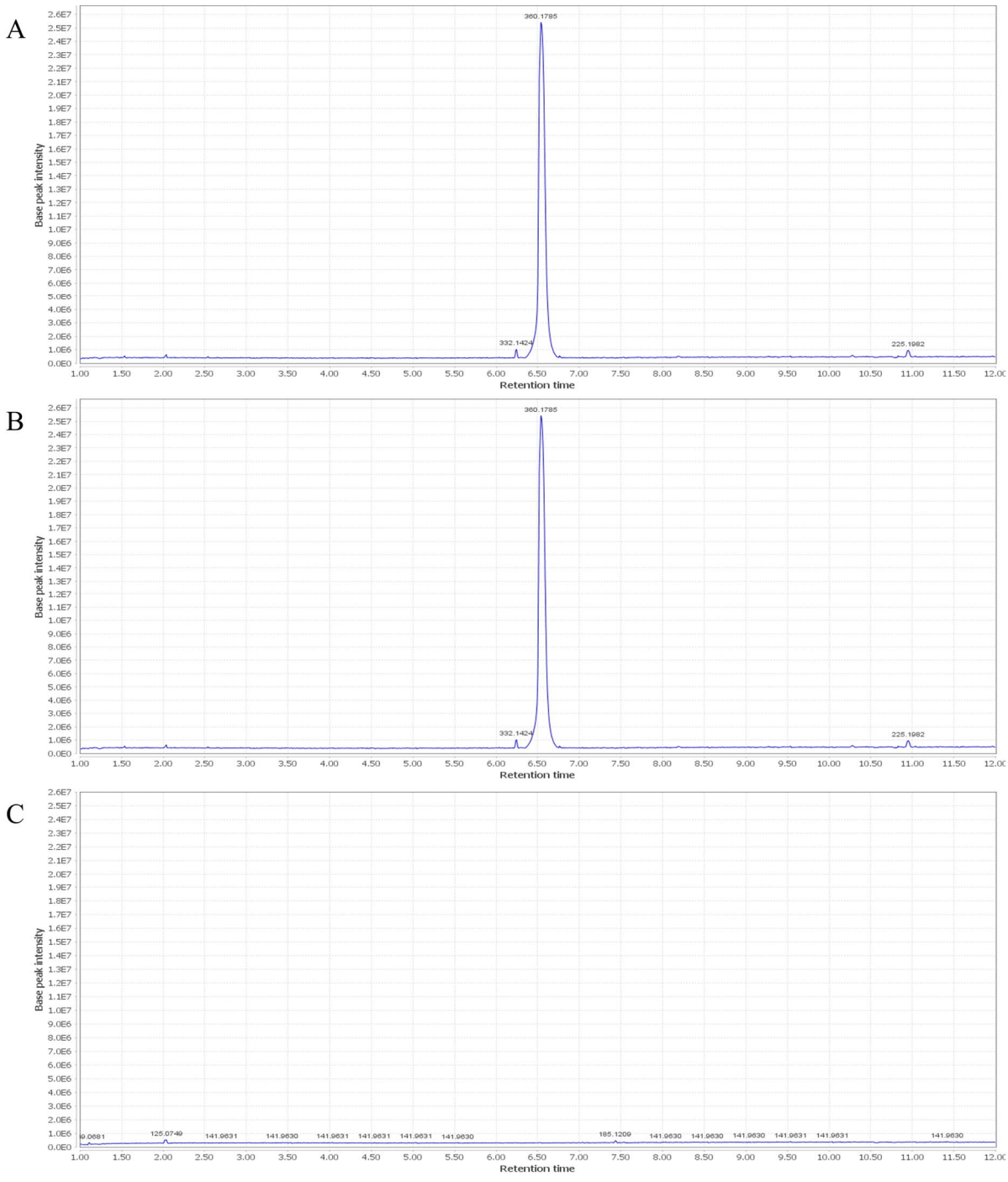


Figure 1: Chemical structure of enrofloxacin; 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid

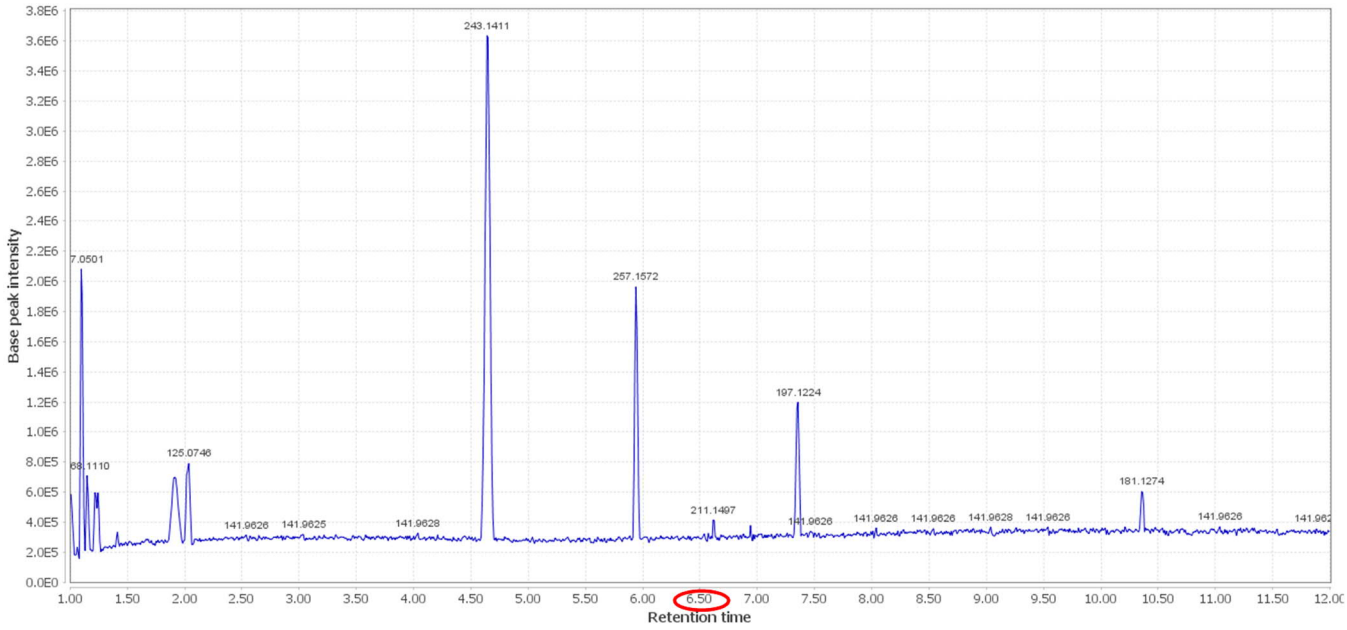


Figure 2: Total ion chromatograms of enrofloxacin (RT 6.5 min; 360 *m/z*) in the control experiments via LC-ToF (ESI (+) MS- full scan mode) analyses; A) the enrofloxacin solution (10 mg l^{-1}) at the start of the experiment, B) the enrofloxacin concentration after 72 h of incubation in the absence of sediment, C) 10 ml water lacking enrofloxacin in sediment after 72 h, D) autoclaved sediments exposed to 10 mg l^{-1} enrofloxacin for 72 h

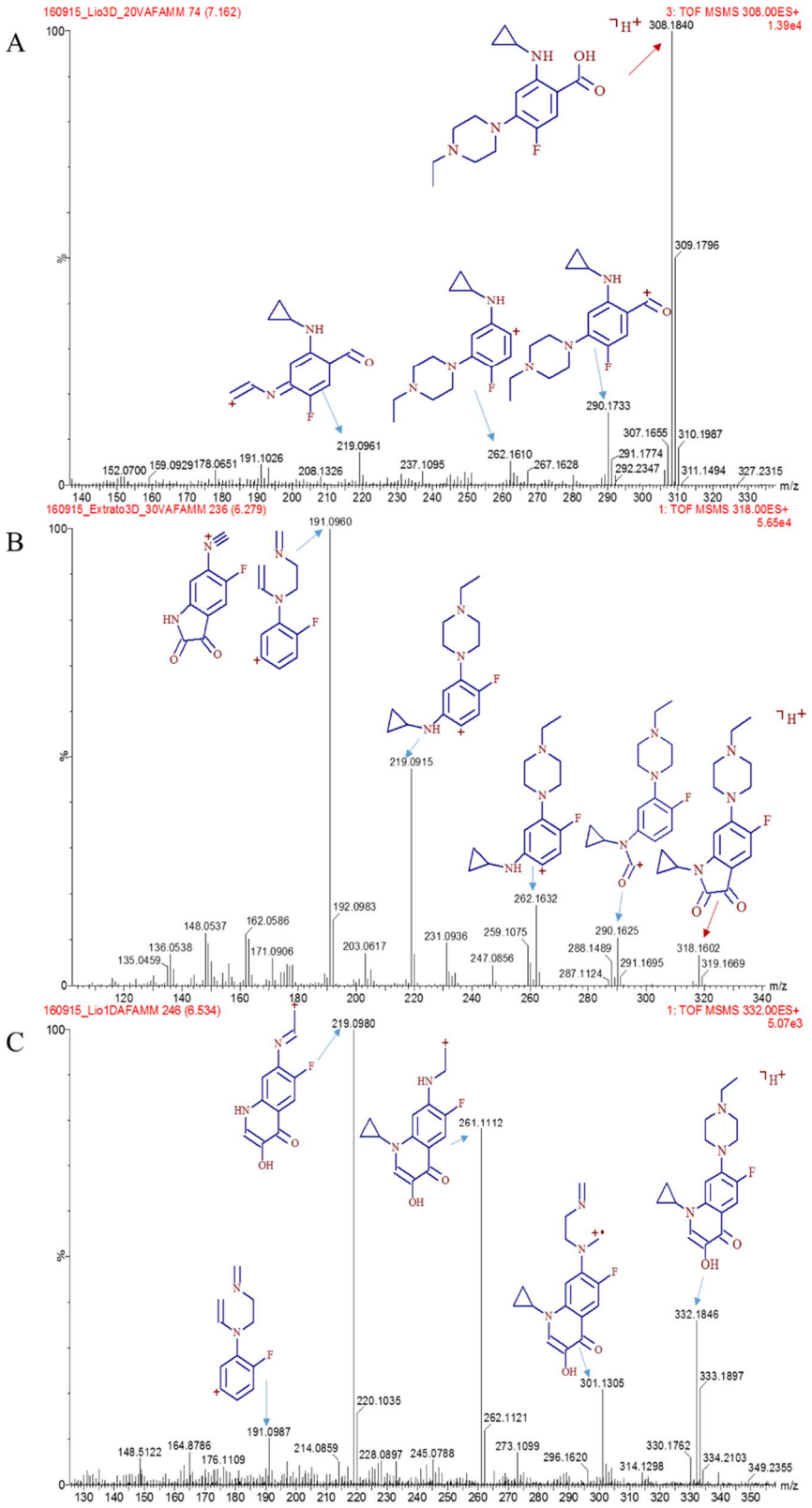
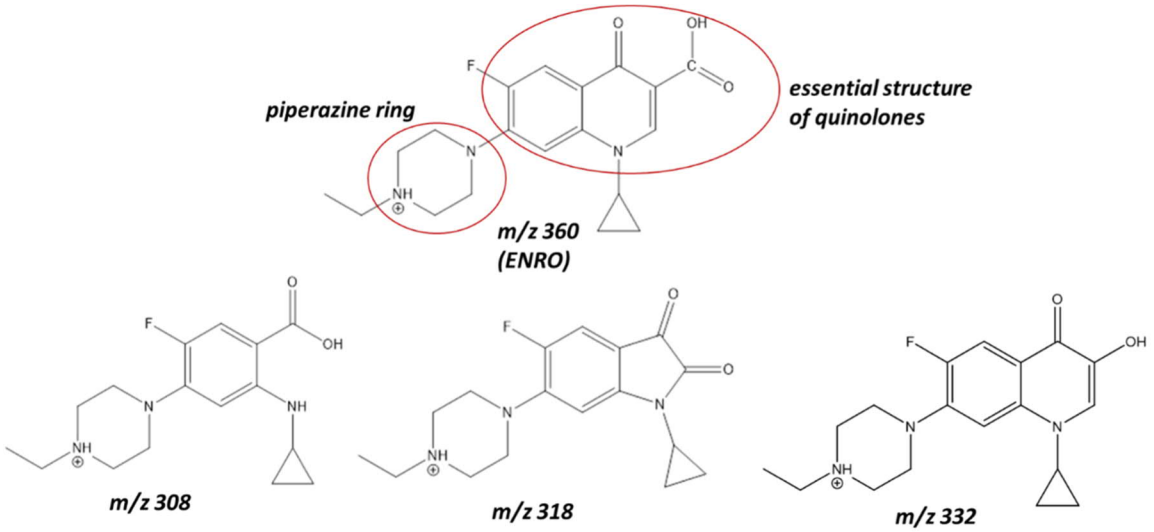
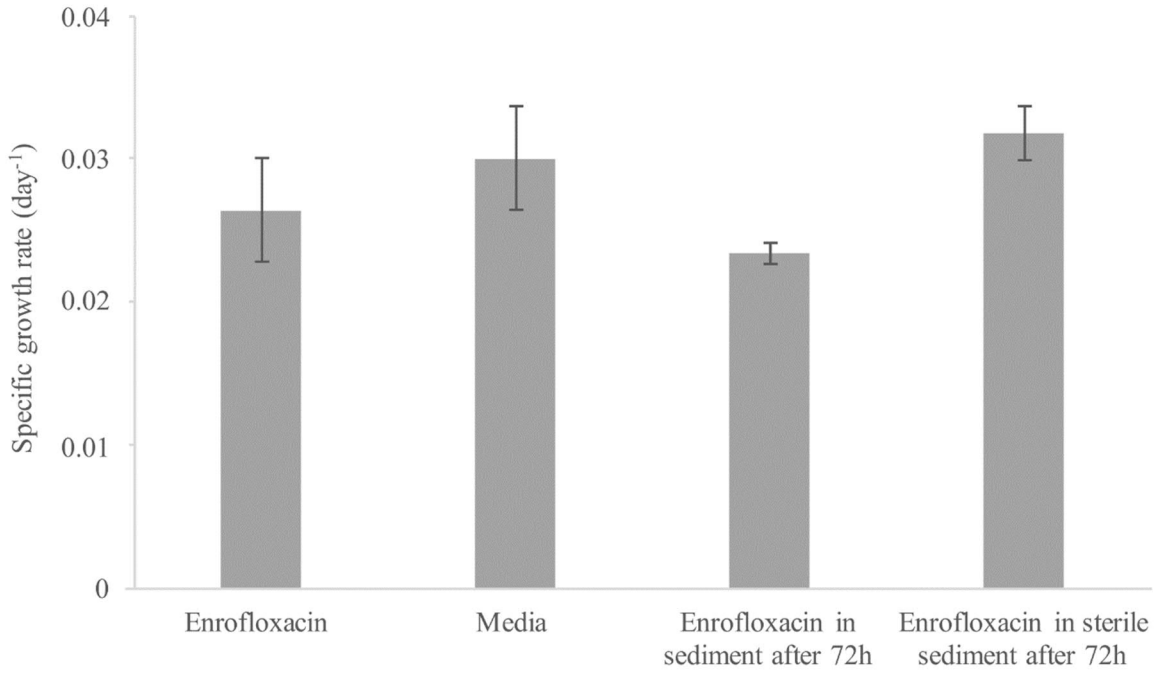


Figure 3: Mass spectrum obtained from analyzes by LC-ToF (ESI (+) MS / MS) after 72 h of exposure with lake sediment for the intermediate structure identification of A) m/z 308, B) m/z 318, and C) m/z 332



356

357 **Figure 4:** The three TPs of enrofloxacin identified in sediment from Müggelsee after 72 h;
358 namely 2-Cyclopropylamino-4-(4-ethyl-1-piperazinyl)-5-fluorobenzoic acid (m/z 308), 1-
359 Cyclopropyl-6-(4-ethyl-1-piperazinyl)-5-fluoro-1H-indole-2,3-dione (m/z 318), and 1-
360 Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-3-hydroxy-4-1H-quinolinone (m/z 332)



361

362 **Figure 5:** Specific growth rate of *Desmodesmus subspicatus* after 72 h of exposure to
363 enrofloxacin at a concentration of 10 mg l^{-1} (positive control), media devoid of enrofloxacin

364 (negative control), the TPs of enrofloxacin after biodegradation in Müggelsee sediment after
365 72 h, and the products of enrofloxacin in sterilized sediment after 72 h. Data represent average
366 μ ± standard deviation (n = 4).

367

368 **Figure 6:** Antibiotic susceptibility test for enrofloxacin biodegradation in sediment from
369 Müggelsee. Enro initial represents an enrofloxacin solution of 10 mg l^{-1}, media was devoid of
370 enrofloxacin (control), the negative control represented media devoid of enrofloxacin
371 incubated in the natural sediment for 72 h, treatment represented media containing 10 mg l^{-1}
372 enrofloxacin incubated with the natural sediment for 72 h, and the positive control represented
373 media containing 10 mg l^{-1} enrofloxacin incubated with the sterilized sediment for 72 h. Data
374 represents the average inhibition zone (mm) ± standard deviation (n = 4)