Environmental Science
Water Research & Technology

Emerging investigator series: Transformation of common antibiotics during water disinfection with chlorine and formation of antibacterially active products

Journal:	*Environmental Science: Water Research & Technology*
Manuscript ID	EW-ART-03-2019-000182.R1
Article Type:	Paper
Date Submitted by the Author:	17-Apr-2019
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This study determined that at chlorination conditions representative of wastewater treatment, fluoroquinolones, one of the common antibiotic classes, form antibacterially active products. Antibacterially active transformation products of antibiotics are a potential contributor to the antibacterial resistance forming in the environment, but are currently not well understood. This study emphasizes their presence in the environmental systems and the water cycle and encourages further investigation into the topic.

Emerging investigator series: Transformation of common antibiotics during water disinfection with chlorine and formation of antibacterially active products.

Nicole L. Kennedy Neth, Clifford M. Carlin, and Olya S. Keen

Abstract:

This study investigated the effect of chlorine disinfection on the following commonly prescribed antibiotics typically present in treated wastewater: ciprofloxacin, trimethoprim, sulfamethoxazole, levofloxacin and ofloxacin. Antibacterially active transformation products formed from all of the fluoroquinolone class antibiotics: ciprofloxacin, levofloxacin and ofloxacin. Trimethoprim and sulfamethoxazole did not form products with detectable antibacterial activity. Experiments were performed in both ultrapure water and wastewater effluent. HPLC/MS was used to propose the structures of the transformation products in samples where antibacterially active products formed. Both chlorinated and non-chlorinated products were observed. The results indicate that antibiotic transformation products with antibacterial properties form in the wastewater chlorination treatment process. These products may be a source of antibiotic resistance in the environment and warrant a further investigation of their role.

Introduction:

Among pharmaceuticals in the environment, antibiotics are of particular interest because of their effect on human health. Several studies have raised a concern about the potential connection between antibiotics in wastewater and the development of antibacterial resistance in environmental microorganisms.[1-5]

When antibiotics go through the wastewater treatment system, chemical reactions can occur that alter their structure creating transformation products. These transformation products may retain their antibacterial potency and are released into the environment with final effluent. [6-8]

Biodegradation is not common for antibiotics[9], and their transformations in wastewater facilities mainly occur when the compounds are exposed to wastewater disinfectants. One of the most commonly used wastewater treatment disinfectants is chlorine. Several studies have already shown that chlorine readily reacts with many pharmaceuticals.[10-14] Previous work by the authors has shown that antibacterially active transformation products form when doxycycline is exposed to chlorine disinfection conditions.[8] These findings prompted this investigation of other common antibiotics and their potential transformation products.

Previous studies have examined degradation of antibiotics during chlorine exposure, with some of the studies focusing on product identification and occasionally product toxicity, but not the antibiotic properties of the products.[14-34] Trimethoprim is among one of the most commonly detected antibiotics in the environment appearing at several hundred ng/L in wastewater effluents.[15-16] Another study has shown that trimethoprim structure was not substantially degraded when reacted with free available chlorine (FAC) even though percent transformation of the parent compound was substantial with conditions typical of wastewater and drinking water chlorination.[14]

Another antibiotic that is commonly prescribed and combined with trimethoprim is sulfamethoxazole.[18] Sulfamethoxazole has been detected at concentrations on the order of 200- 2000 ng/L in wastewater effluents.[19-21] Some studies have shown that

sulfamethoxazole is reactive with free chlorine but did not identify transformation products.[22-23] Other studies with sulfamethoxazole and FAC identified products, but the antibacterial activity of those products have not been examined.[24-26]

Fluoroquinolones are a class of antibiotics of particular interest because they are not completely metabolized, and for this reason, a substantial amount can be discharged into wastewater treatment facilities. Studies have reported fluoroquinolones present in wastewater effluents at concentrations in the range of 70 – 500 ng/L.[27-29] Previous studies reported a number of transformation products for ciprofloxacin, although their bactericidal activity was not investigated.[30,31] A recent study investigated ciprofloxacin chlorinated products and their effects in model drinking water distribution systems.[32] The results indicated that the increase of antibiotic resistance genes detected in the study was from the growth of bacteria in the presence of the chlorinated transformation products, although the results were not verified for actual distribution systems yet.[32]

A recent study investigated the chlorination process with levofloxacin, a commonly prescribed fluoroquinolone antibiotic, and found four transformation products.[33] The early transformation products showed more toxicity than the parent compound to *V. fisheri*. Another study examined the reaction between FAC and ofloxacin. Ofloxacin is another representative of the fluoroquinolone class of antibiotics and is a racemic mixture of levofloxacin and dextrofloxacin. Several transformation products were reported including some chlorinated transformation products.[34]

This study comprehensively evaluated the effects of chlorine disinfection on antibacterial properties of transformation products from several antibiotics commonly

prescribed and frequently detected in waterways. Potentially active products were identified using established structure-activity relationships.

Experimental (Materials and Methods):

The antibiotics selected for the study represent a dihydrofolate reductase/sulfonamide class (trimethoprim/sulfamethoxazole) and fluoroquinolones (ciprofloxacin, levofloxacin, and ofloxacin). Ciprofloxacin, sulfamethoxazole, trimethoprim, 10-15% reagent grade solution of sodium hypochlorite and 30% reagent grade solution of hydrogen peroxide were obtained from Sigma-Aldrich (St. Louis, MO). Ciprofloxacin, trimethoprim, and sulfamethoxazole were initially selected for examination as some of the antibiotics most commonly prescribed and detected in water resources. After initial experiments showed antibacterially active products of ciprofloxacin, more of the common fluoroquinolone class antibiotics (levofloxacin and ofloxacin) were tested. Levofloxacin (98% powder) and ofloxacin (98.5% powder) were obtained from Alfa Aesar (Ward Hill, MA). Ofloxacin is a racemic mixture of levofloxacin (active component) and dextrofloxacin (8-128 times lower activity than levofloxacin).[35]

UV-disinfected wastewater effluent was collected from a local wastewater treatment plant and filtered within hours of collection through a nylon 0.8 μm membrane filter (Whatman, Piscataway, NJ) followed by a nylon 0.2 μm membrane filter (Merck Millipore Ltd., Billerica, MA) to remove any microorganisms that would compete with the test organism, *Escherichia coli* (ATCC 11303), in the antibacterial activity assay. After filtration the samples were kept at 4 °C and brought to room temperature before use. The samples were redisinfected just prior to experimentation using a benchtop low

92 pressure mercury lamp collimated UV beam at the dose of 250 mJ/cm^2. Additionally,
93 control assays were performed with the wastewater matrix to verify no indigenous
94 growth.
95 Water quality parameters for the wastewater sample indicated nitrite at <0.015
96 mg-N/L (nitrite HACH test TNT 839), nitrate at 21.2 mg-N/L (nitrate HACH test TNT
97 835), and ammonia at <0.1 mg/L NH_3-N (ammonia HACH test TNT 831). The total
98 organic carbon was 6.8 mg/L and was measured using a Shimadzu TOC-LCPN. The
99 absorbance for the water samples at 254 nm was 0.11 and was measured using a HACH
100 DR 6000 UV-Vis Spectrophotometer. The pH was 7.0 (HACH pH meter H280G) and the
101 alkalinity was 42.4 mg/L as $CaCO_3$ (HACH alkalinity digital titrator, AL-DT).
102
103 *Chlorination Procedure:* Ciprofloxacin solution with concentration of 2.33 mg/L,
104 trimethoprim solution with concentration of 20 mg/L, sulfamethoxazole solution with
105 concentration of 2 mg/L, sulfamethoxazole in tandem with trimethoprim at the
106 prescription 5:1 ratio (to test synergistic effects) solution with concentration of 2 mg/L
107 and 0.4 mg/L respectively, levofloxacin solution with concentration of 2 mg/L, and
108 ofloxacin solution with concentration of 2 mg/L were prepared in ultrapure water
109 (Thermo Scientific Barnstead Nanopure Diamond water purification system, 18 MΩ·cm,
110 < 0.5 mg/L TOC) or in effluent. Preliminary experiments were performed to determine
111 these optimal concentrations to be used in order for the antibiotics to be effective in the
112 assay and to improve the detection of the products. Any background antibiotics that may
113 be present in the effluent would have concentration orders of magnitude lower and would
114 not interfere with the assay. Ultrapure water was used for detection of products to ensure

115 no interferences from the sample matrix.

116 A sample was taken before chlorine was added and at the time intervals of 0.5, 1,
117 5, 10, 30, 60 and 120 min of chlorine exposure. At each time interval, a sample was taken
118 for high performance liquid chromatography with mass spectrometry (HPLC/MS)
119 analysis as well as for antibacterial activity assays. To ensure chlorine was present
120 throughout the duration of the experiments, the initial chlorine dose used was determined
121 with a goal of no less than 0.2 mg/L of residual Cl_2 as free chlorine at the end of the 120
122 min. The chlorine concentrations were measured using a HACH DR 2800
123 spectrophotometer (Hach Corporation, Loveland, CO) with *N,N*-diethyl-p-
124 phenylenediamine colorimetric method (HACH DPD free chlorine powder pillows).

125 Once the sample was transferred into vials for further testing at each specified
126 time interval, the residual chlorine was quenched with hydrogen peroxide (H_2O_2). The
127 H_2O_2 instantly reacted with chlorine[7] and was chosen over other reagents, such as sodium
128 sulfite or sodium thiosulfate because H_2O_2 does not add background levels of inorganics,
129 which can interfere with the mass spectrometry instrumentation. Preliminary experiments
130 were performed on the antibiotics and H_2O_2 to assure that no reaction between the
131 antibiotics and H_2O_2 took place and no intermediates formed. While H_2O_2 is commonly
132 considered a strong oxidant, its reactivity with organic compounds typically requires high
133 concentrations, high temperature and alkaline pH, therefore, no reaction with
134 transformation products was expected either. The concentration of H_2O_2 used was
135 determined by the stoichiometric ratio for chlorine quenching (1 mg/L of H_2O_2 to 2.1
136 mg/L of Cl_2). To ensure that residual H_2O_2 did not affect the assays, the samples were
137 further quenched with bovine catalase (Sigma-Aldrich, St. Louis, MO) at a dose of 1

mg/L with at least 30 minutes reaction time as done in previous studies.[7,8] Another control experiment was performed with the chlorine-treated wastewater effluent (with no antibiotics added and with chlorine quenched) to make sure there was no interference from the effluent matrix with the bacterial growth in the assay (either promoting or inhibiting growth). The growth in these controls corresponded to the growth in positive controls.

Antibacterial Activity Assay: The chosen antibiotics are effective against gram-negative cells, so a non-resistant strain of *Escherichia coli* (ATCC 11303) was used as a test organism in the antibacterial activity assays. The bacterial culture was grown at 37 °C in sterile broth in a shaking incubator until cloudy (approximately 24 h). The broth consisted of 500 mL of ultrapure water, 5 g of tryptone (Fisher Scientific, Fair Lawn, NJ), 2.5 g of yeast extract (Fisher Scientific, Fair Lawn, NJ) and 2.5 g of sodium chloride (Fisher Scientific, Fair Lawn, NJ). One mL of the 24-hour culture was transferred to 50 mL of fresh sterile broth and incubated for approximately 1 h to the optical density (absorbance) at 600 nm (OD_{600}) of 0.20 +/- 0.03. The OD_{600} was measured using the HACH DR6000 spectrophotometer. The 1 h culture was then diluted by a factor of 10 with sterile broth to achieve the cell concentration of approximately 10^6 cells/mL to be used in the assays. The correlation between OD_{600} and cell count for this strain of *E. coli* was determined previously and incorporated into the assay protocol that was adopted for this study.[7]

A phosphate-buffered saline (PBS) was prepared in the lab and autoclave sterilized. PBS recipe consisted of 3.2 g of sodium chloride, 0.08 g of potassium

chloride, 0.72 g of dibasic sodium phosphate dihydrate, and 0.1 g of monobasic potassium phosphate diluted in 400 mL of ultrapure water. Monobasic potassium phosphate was purchased from Sigma Aldrich (St. Louis, MO) with the rest of the chemicals obtained from Fisher Scientific (Fair Lawn, NJ). PBS was used for a serial factor-of-2 dilution of the samples in the assay, and 100 µL was used around the perimeter of the assay plates for evaporation control due to the duration (4 h) of the experiment. The dilutions were prepared in a flat-bottom, non-treated sterile Cellstar 96-well plate (Greiner Bio-one, Monroe, NC). The negative control consisted of the 100 µL of PBS and 100 µL of sterile broth and was not expected to exhibit growth. The negative control was used to monitor if contamination occurred in the sterile solutions or in the assay. The positive control contained 100 µL of PBS and 100 µL of bacterial culture and was used to measure bacterial growth not inhibited by antibiotics.

After dilutions were prepared, 100 µL of a 1 h *E. coli* culture was added to each sample well and the positive control. Growth in the sample wells was calculated as the percentage of growth in the positive controls. A sterility control with UV-pre-disinfected wastewater effluent was conducted using the same conditions as the actual assay and showed a change in OD_{600} after the 4 h incubation of 0.002 ± 0.003 (average and standard deviation of 60 wells). This represents the noise of the instrument and is consistent with negative controls for the assays. In contrast, the OD_{600} of positive controls was on the order of 0.24. The OD_{600} readings of the wells was measured using a Bio-Tek Instruments µQuant microplate reader model MQX200 (Winooski, VT) at 600 nm. Readings were taken before the assays were incubated and immediately following their 4 h incubation period at 37 °C. The 4 h incubation period was selected in order for the bacteria to be in

their exponential growth phase and for the OD_{600} readings to be above 0.1 in the positive controls.

To determine the LD_{50} for each sample, the data was linearized using Probit analysis[36] by plotting Probit values corresponding to the observed % growth against the log of concentration. The LD_{50} was calculated from the linear regression as the concentration of the antibiotic at which the bacterial growth in the sample was 50% of the growth in the positive control. LD_{50} of the untreated sample divided by the LD_{50} of the treated sample was designated as potency equivalent (PEQ)[14] for each sample. Increased antibiotic potency of the sample is associated with decreased growth in the assays. The concentration of the parent antibiotic remaining (measured by HPLC/MS analysis) was compared to the PEQ of each sample. When the PEQ values were higher than the fraction of the parent antibiotic remaining, it indicated that new antibiotics have formed. The chlorination experiments were performed three times in full replication, each replicate including duplicate assays

HPLC/MS methods: Vanquish flex quaternary ultrahigh performance liquid chromatography system and a Velos pro dual-pressure linear ion trap mass spectrometer with electrospray ionization (ESI) source were used for analysis of the structure of the products and fraction of antibiotic remaining. The method was run in positive ionization mode with a full scan of m/z range 200-1000. The mobile phase consisted of solvent A (HPLC grade water with 0.1% formic acid) and solvent B (HPLC grade acetonitrile with 0.1% formic acid). The gradient began with a 1 min delay during which the flow was diverted to waste to minimize the potential contamination of the mass spectrometer by inorganic wastewater effluent constituents. This was followed by a 15 min ramp from

10% to 100% solvent B (for sulfamethoxazole, trimethoprim and ciprofloxacin) or a 20 min ramp (for levofloxacin and ofloxacin), 1 min flush at 100% solvent B, equilibration to 10% solvent B, and a 2 min relaxation before next injection. The injection volume was 10 μL, the mobile phase flow was maintained at 0.4 mL/min, and the column temperature was 35 °C. The column was Hypersil GOLD C_8, 100 x 2.1 mm with 3 μm particle size (Thermo Scientific, Waltham, MA).

Results and discussion:

The concentration×time (CT) values were consistent with those used in wastewater disinfection (0.6-192).[37] The CT values for the antibiotics can be seen in Table S1 for both ultrapure water and wastewater at 10 min and 120 min and are based on chlorine residuals measured at each sampling point up to that time (e.g. at 0.5, 1, 5 and 10 min for the CT at 10 min). The initial value of chlorine spike differed in ultrapure water and wastewater to achieve approximately the same residual. Higher concentrations of Cl_2 were used for wastewater experiments because of increased chlorine demand by the wastewater constituents. Chlorine was measured at each time point during preliminary experiments and the residual values at the end of each experiment can be seen in Table S2 as well as the initial chlorine and antibiotic concentrations for all antibiotics in both ultrapure water and wastewater. In most instances, the reaction rate for each antibiotic with chlorine was either similar in ultrapure water and in wastewater matrix or slower, accounting for the competing reactions between chlorine and organic matter. The only exception was ciprofloxacin, which reacted with chlorine faster in wastewater matrix than in ultrapure water matrix. Ciprofloxacin solution required a higher increase in chlorine dose to maintain a residual in wastewater than in ultrapure water compared to other

antibiotics (3.25 times more chlorine in wastewater effluent compared to ultrapure for ciprofloxacin, compared to 2-2.5 times for other antibiotics). It was in general more reactive with chlorine than the other antibiotics tested, and its unique competition reactions with organic matter may have resulted in the required higher chlorine dosage and the observed increased reaction rate in wastewater effluent matrix.

All the antibiotics were dissolved in ultrapure water and adjusted to a pH of approximately 6.95 – 7.25 using either hydrochloric acid (Fisher Scientific, Hampton, NH) or sodium hydroxide (Sigma Aldrich, St. Louis, MO). The pH remained stable with all the antibiotics within this range for wastewater and no future pH adjustment was needed. The pH also remained within this range for the duration of the experiments. The pK_a values of the antibiotics chosen are listed in Table S3, and the hypochlorous acid pK_a is 7.6.[38] Trimethoprim, levofloxacin and ofloxacin have pK_a values sufficiently close to pH 7, thus the experiments captured the species that would be present within the range of pH typical for wastewater, drinking water, and natural waters (pH 6-8). Sulfamethoxazole and ciprofloxacin have pK_a values that are outside of this range.

Apart from individual results, sulfamethoxazole and trimethoprim were also combined in their prescribed dosage of 5:1 ratio to test whether the synergetic relationship between the two antibiotics has significance for transformation products (TPs). No antibacterially active transformation products were detected for sulfamethoxazole, trimethoprim, or the combined sulfamethoxazole/trimethoprim experiments. Therefore, detailed MS analysis of the products was not performed. While sulfamethoxazole and trimethoprim work in tandem as antibiotics, no synergistic effects were detected for the TPs. The results for these experiments can be seen in Figure 1.

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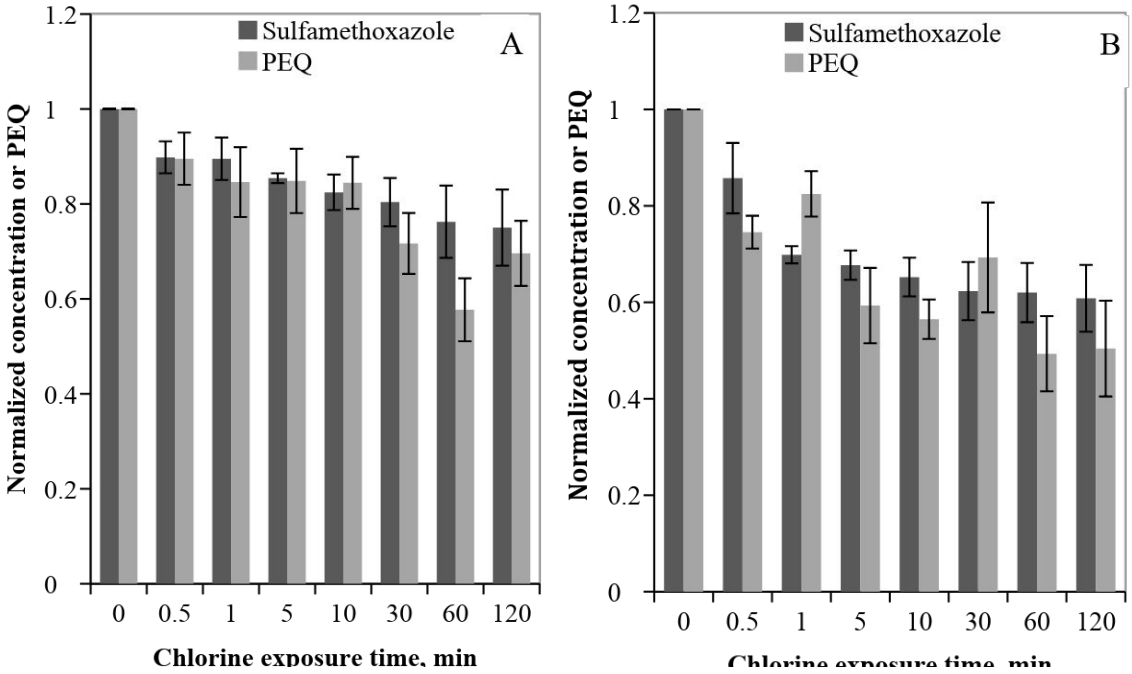

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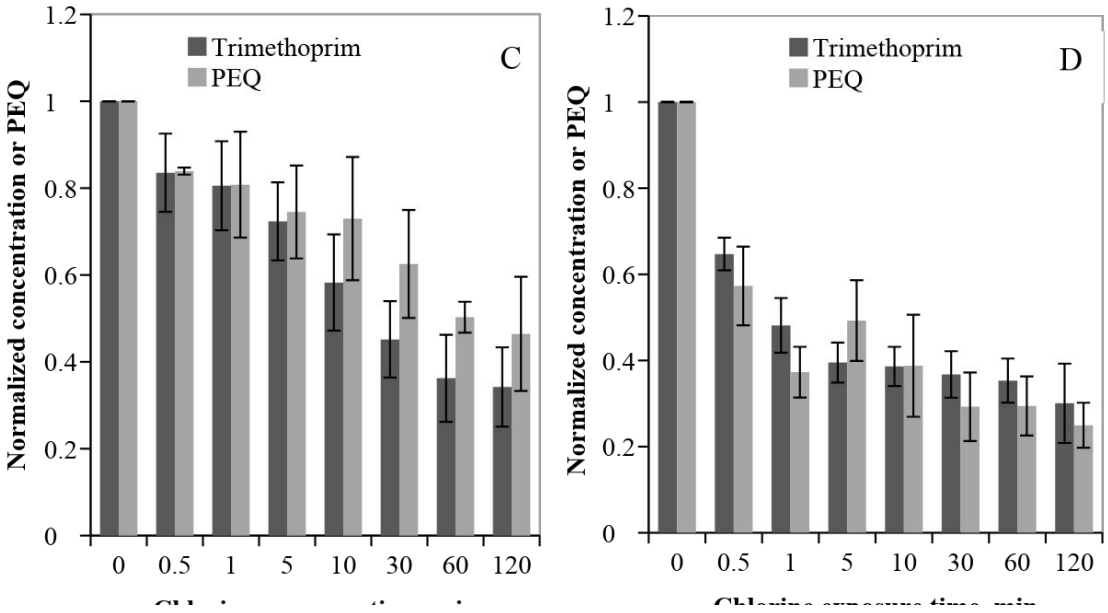

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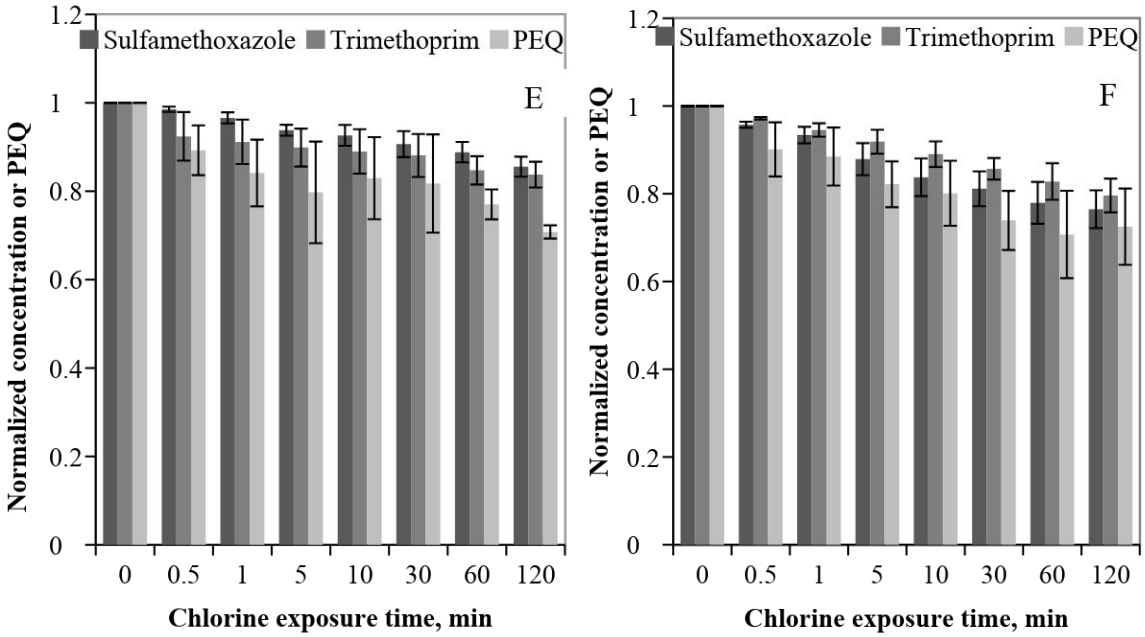


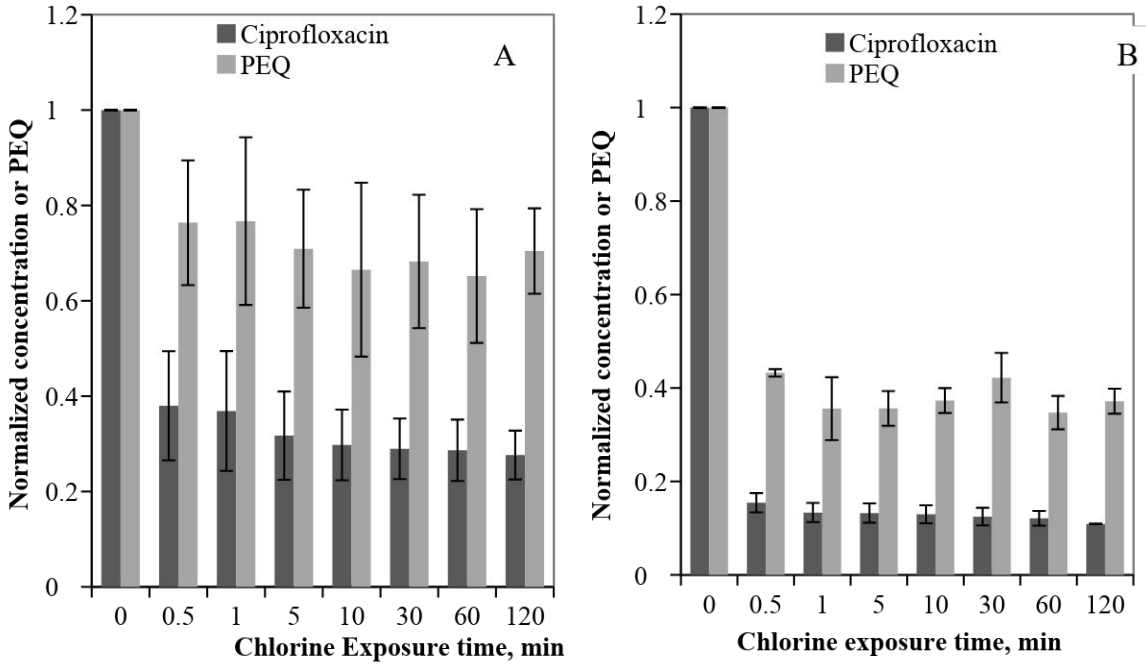
Figure 1. PEQ vs. normalized concentration of sulfamethaxozole in ultrapure matrix (A) and wastewater effluent matrix (B), trimethoprim in ultrapure matrix (C) and wastewater effluent matrix (D), sulfamethaxozole in tandem with trimethoprim in ultrapure matrix (E) and wastewater effluent matrix (F) after specific time intervals of chlorine exposure. Results are based on averages from three sets of replicated experiments. Error bars represent standard deviation from three repeated experiments

Experiments with ciprofloxacin showed that TPs retained antibiotic potency (Figure 2). Active products were present at every exposure time in both ultrapure water and wastewater (Figure 2). Results for levofloxacin and ofloxacin, both of which also displayed formation of antibacterially active products, can be seen in Figure 2 as well. Active TPs were detected in both ultrapure water and wastewater for the experiments with levofloxacin. The formation of active products from levofloxacin increased with

longer exposure times in ultrapure water. This is probably related to the higher degree of parent molecule transformation achieved in ultrapure water (Figure 2). Active TPs formed in both ultrapure water and wastewater for the experiments with ofloxacin as well. The active products of ofloxacin appear to form with longer exposure times in wastewater matrix (Figure 2).

The following bimolecular rate constants are reported in literature for the antibiotics in this study: 2,000 M^{-1} s^{-1} for sulfamethoxazole,[24] 56 M^{-1} s^{-1} for trimethoprim,[14] 10^5-10^6 M^{-1} s^{-1} for ciprofloxacin,[30] 4,400 M^{-1} s^{-1} for levofloxacin,[33] and 6,800 M^{-1} s^{-1} for ofloxacin,[34] all at near neutral pH. The experiments in this study were not designed to measure reaction rate constants, and both reactants were allowed to change in concentration through the experiment. However, the initial observed rate of the reaction in the first 30 s was on the order of magnitude with the predicted rate based on the reaction rate constants reported in literature for all antibiotics except ciprofloxacin. The closest match to the predicted value was for trimethoprim with the observed initial reaction rate within 40% of the predicted rate. The observed initial rate for ciprofloxacin was two orders of magnitude slower than the predicted value based on literature reports. It appears from the data that the reaction of fluoroquinolones, especially ciprofloxacin, with chlorine proceeds rapidly in the first 30 s after which it slows down. This may be indicative of a higher order reaction with respect to one or both of the reactants where small changes in concentrations cause a significant decrease in the observed reaction rate. Degradation products of ciprofloxacin may also be reactive with chlorine at a higher rate than the parent compound, and can provide significant competition as they build up in the process. The change in reaction rate through the experiment was unexpected and

297 warrants further investigation. However, the antibacterial properties of the
298 transformation products, which were the focus of this study, were not impacted by the
299 reaction rate, but rather by the degree of transformation of the parent compound.
300 Ofloxacin and levofloxacin were transformed to a lesser degree in the effluent
301 matrix compared to the ultrapure water matrix, potentially due to chlorine scavenging
302 reactions of the background organic matter. However, the results were the opposite for
303 ciprofloxacin. This could be due to two reasons. First, chlorine concentration necessary
304 to achieve the desired two-hour residual was determined in preliminary experiments for
305 each of the antibiotics and was based on the individual competition kinetics between the
306 antibiotic and the background organic matter. Chlorine dose also depended on the initial
307 concentration of the antibiotic used, as those varied depending on their antibacterial
308 activity (higher concentration of less potent antibiotics was necessary for the assays). For
309 levofloxacin and ofloxacin, the initial concentration of chlorine in the effluent matrix was
310 1.9 times the concentration used in the ultrapure water. For ciprofloxacin that ratio was
311 3.3 to achieve the same residual chlorine. Second, ciprofloxacin may be reactive with
312 intermediates generated in one of the background chlorine reactions. As this study
313 determined the products that formed in all of the reactions involved in chlorination of
314 ciprofloxacin in effluent, no further experiments were performed to determine specific
315 reactions.
316


Ciprofloxacin
PEQ
A
Normalized concentration or PEQ
Chlorine Exposure time, min
Ciprofloxacin
PEQ
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Normalized concentration or PEQ
Chlorine exposure time, min

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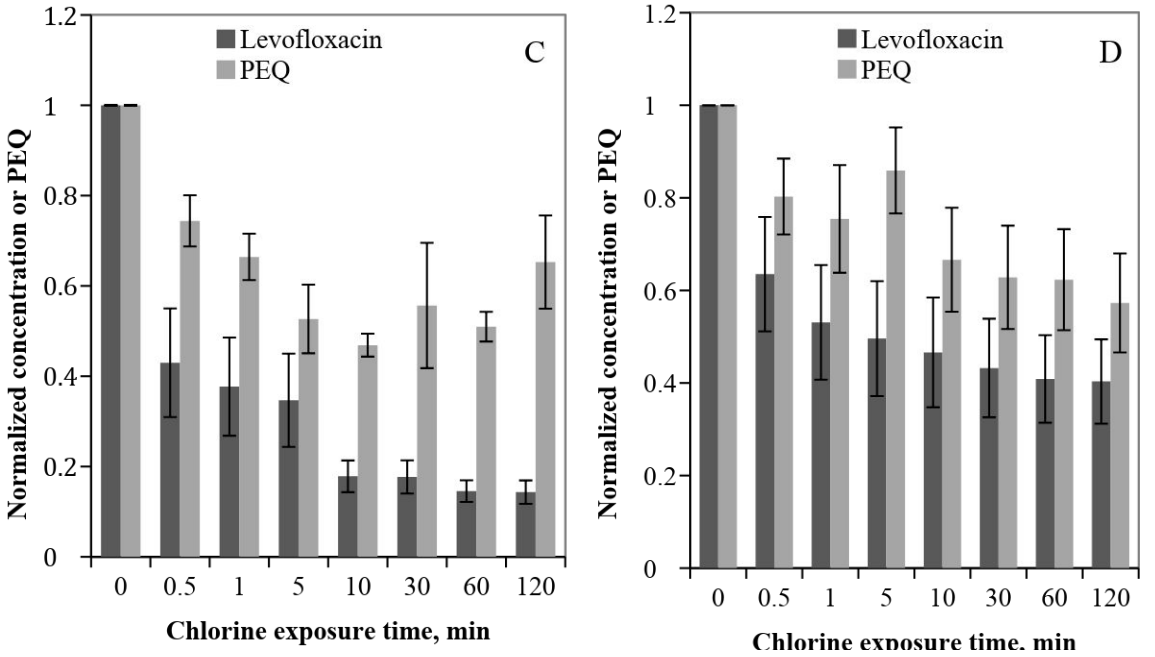
Levofloxacin
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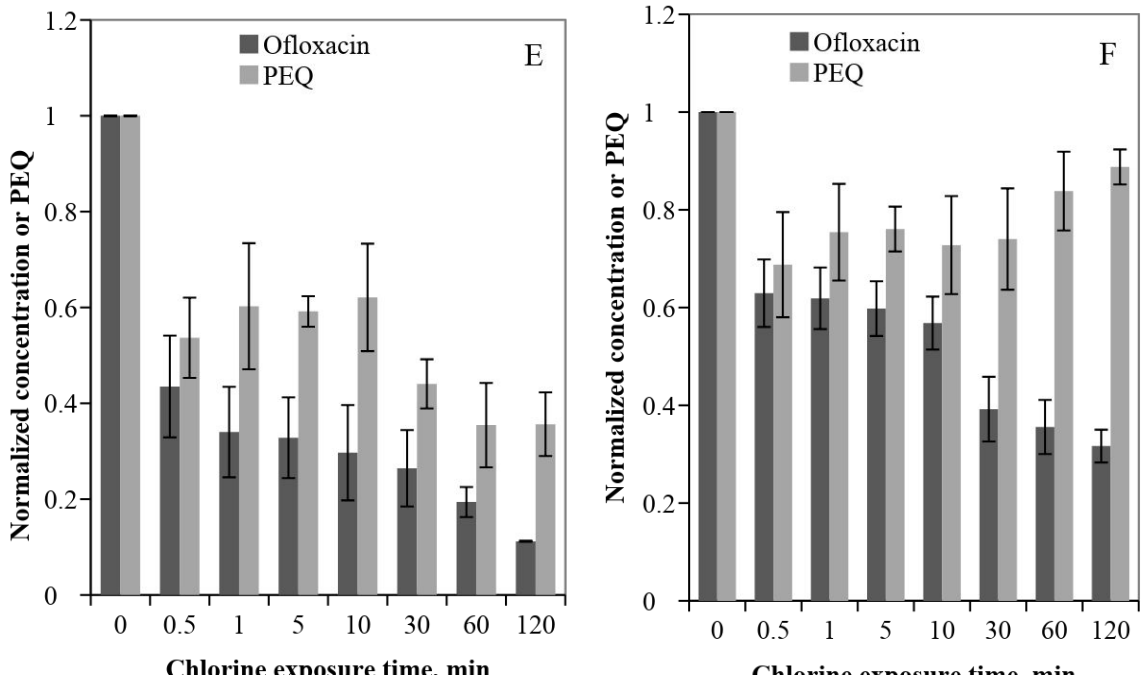

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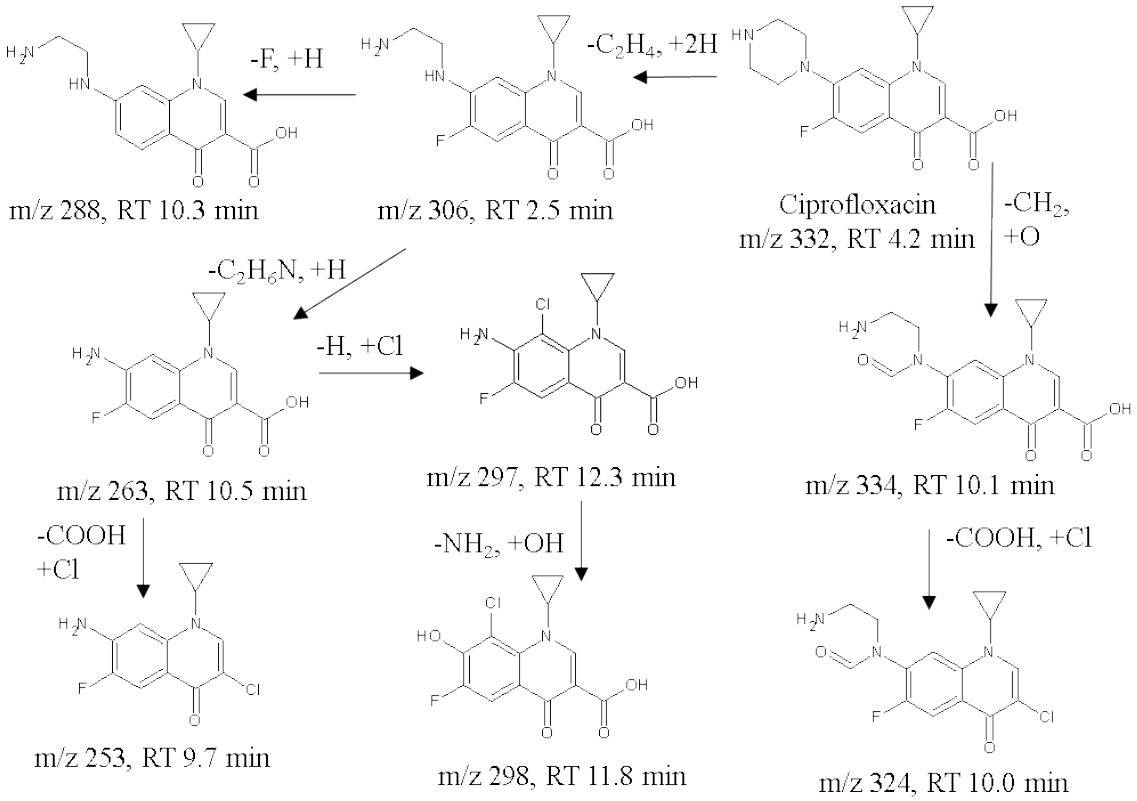
322 **Figure 2.** PEQ vs. normalized concentration of ciprofloxacin in ultrapure matrix (A) and
323 wastewater effluent matrix (B), levofloxacin in ultrapure matrix (C) and wastewater
324 effluent matrix (D), and ofloxacin in ultrapure matrix (E) and wastewater effluent matrix
325 (F) after specific time intervals of chlorine exposure. Results are averages of data from
326 three sets of replicated experiments. Error bars represent standard deviation.

327

328 Ofloxacin is a racemic mixture of the enantiomers of levofloxacin and
329 dextrofloxacin. Levofloxacin is the more biologically active enantiomer while
330 dextrofloxacin has significantly lower biological activity. With dextrofloxacin being an
331 enantiomer of levofloxacin, the corresponding TPs will be enantiomers (unless the chiral
332 center is lost in a reaction) and can potentially have different antibacterial activity.
333 Levofloxacin and dextrofloxacin differ only by the chirality of the carbon to which the

methyl is attached (Table S4 shows the structures). Although the products of levofloxacin and ofloxacin may have different activities, any enantiomers will not be distinguishable from each other using mass spectrometry analysis without the use of a chiral column. Different trends in active product formation for levofloxacin and ofloxacin suggest that dextrofloxacin is capable of forming active transformation products.

Eight major products of ciprofloxacin formed (four of them chlorinated). Their probable reaction pathways and chlorine isotope identification were used to propose the structures of transformation products (Figure 3). The products had *m/z* values (protonated masses) in order of retention time of 306 (non-chlorinated), 253 (mono-chlorinated), 324 (mono-chlorinated), 334 (non-chlorinated), 288 (non-chlorinated), 263 (non-chlorinated), 298 (mono-chlorinated), and 297 (mono-chlorinated).



346

347 **Figure 3.** Chemical structures of the transformation products and parent compound,

348 ciprofloxacin, with their corresponding m/z values and retention time (RT).

349

350 The following are the proposed reaction pathways for these transformation products. The

351 product *m/z* 306 forms when CIP loses two carbons and two hydrogens from its

352 piperazinyl ring. The *m/z* 306 structure further loses two carbons, five hydrogens, and a

353 nitrogen from the remainder of the piperazinyl ring to form the TP with *m/z* 263 where

354 most of the piperazinyl ring is released, leaving an amine group. The *m/z* 306 and *m/z* 263

355 have been reported in previous studies with chlorination, demonstrating that piperazinyl

356 ring is a fragile moiety of the ciprofloxacin molecule. [30,32] Alternatively, *m/z* 306 can lose

357 the fluorine atom to form a product with *m/z* 288.[32] The product with *m/z* 263 can further

358 react by two different pathways: (1) by chlorination of the aromatic ring to form a

product with *m/z* 297;[30] or (2) by substitution of the carboxyl group with chlorine (this reaction was shown for another fluoroquinolone enrofloxacin[30]) (product with *m/z* 253). The *m/z* 297 product can further react by substitution of the primary aromatic amine with a hydroxyl group[31] (*m/z* 298). Yet another pathway involves a formation of an intermediate product where the piperazinyl ring is opened and a double-bonded oxygen adds to it (*m/z* 334)[32]. On that product, the carboxyl group is further substituted by a chorine to form a product with *m/z* 324. This halodecarboxilation reaction was previously reported for enrofloxacin but not for ciprofloxacin[30]. The reaction may be catalyzed by an unstable reactive chlorammonium intermediate.[30] Additionally, this reaction may be the result of homolytic cleavage of chlorammonium yielding chlorine radical.[30] In the study by Dodd et al. (2005) that discusses this mechanism in detail, methanol present in the samples would have scavenged the chlorine radical and prevented this pathway from being considerable.[30] In this study, however, in the absence of a strong competitor for chlorine radical reaction, halodecarboxylation of ciprofloxacin may have resulted despite not being observed in the prior work. Prior research with ciprofloxacin demonstrated formation of various chlorinated products with chlorine attachment on the aromatic ring or the remaining portion of the open piperazinyl ring.[30] Loss of the carboxyl group or its substitution by chlorine has not been reported for ciprofloxacin before, but was reported for other fluoroquinolone antibiotics suggesting the vulnerability of this functional group.[33,34] Additionally, substitution of the carboxyl group with a hydroxyl group was reported for chlorination of ciprofloxacin.[32]

Figure 4 shows the trends in product formation over time. Products with *m/z* 334 and 306 decay over time, confirming that those products are intermediates for future

transformations. Product with *m/z* 288 increases for some time, and then begins to decrease. However, its subsequent products were not detected. It is likely that it proceeds with transformations on piperazinyl ring. The products that form last are those with *m/z* 253, 297 and 298, which is consistent with the pathway outlined in Figure 3.

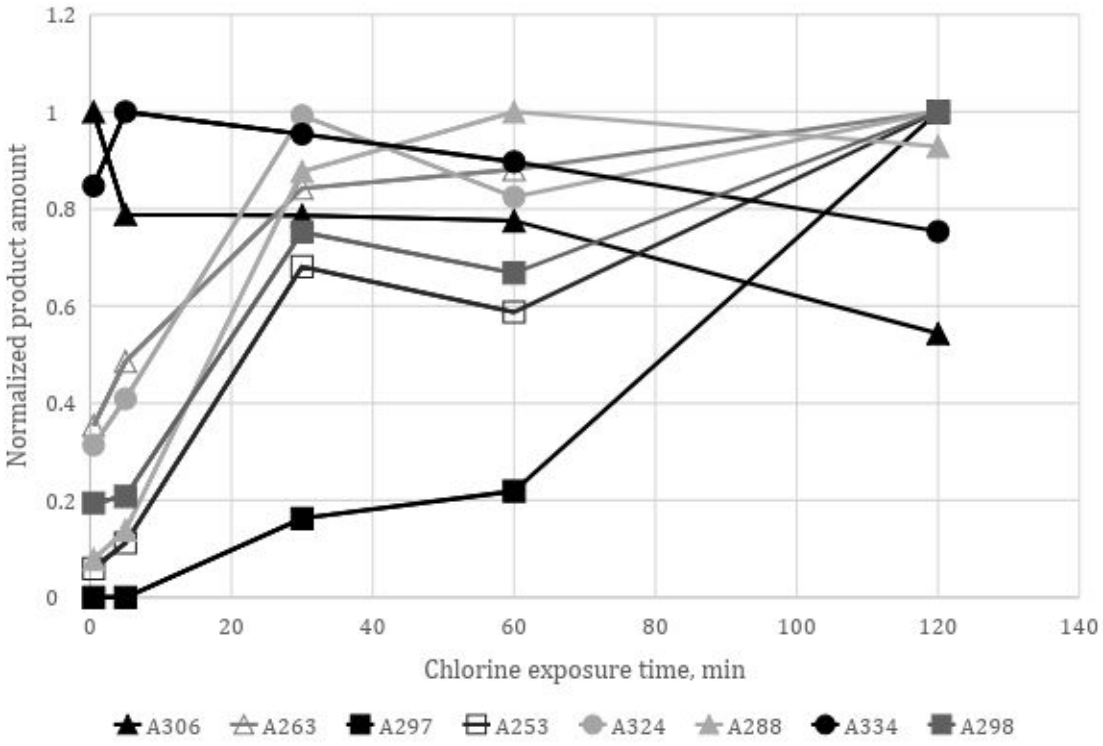


Figure 4: Formation trends of transformation products of ciprofloxacin over time (lines added for visualization). First data point is at 1 min after chlorination.

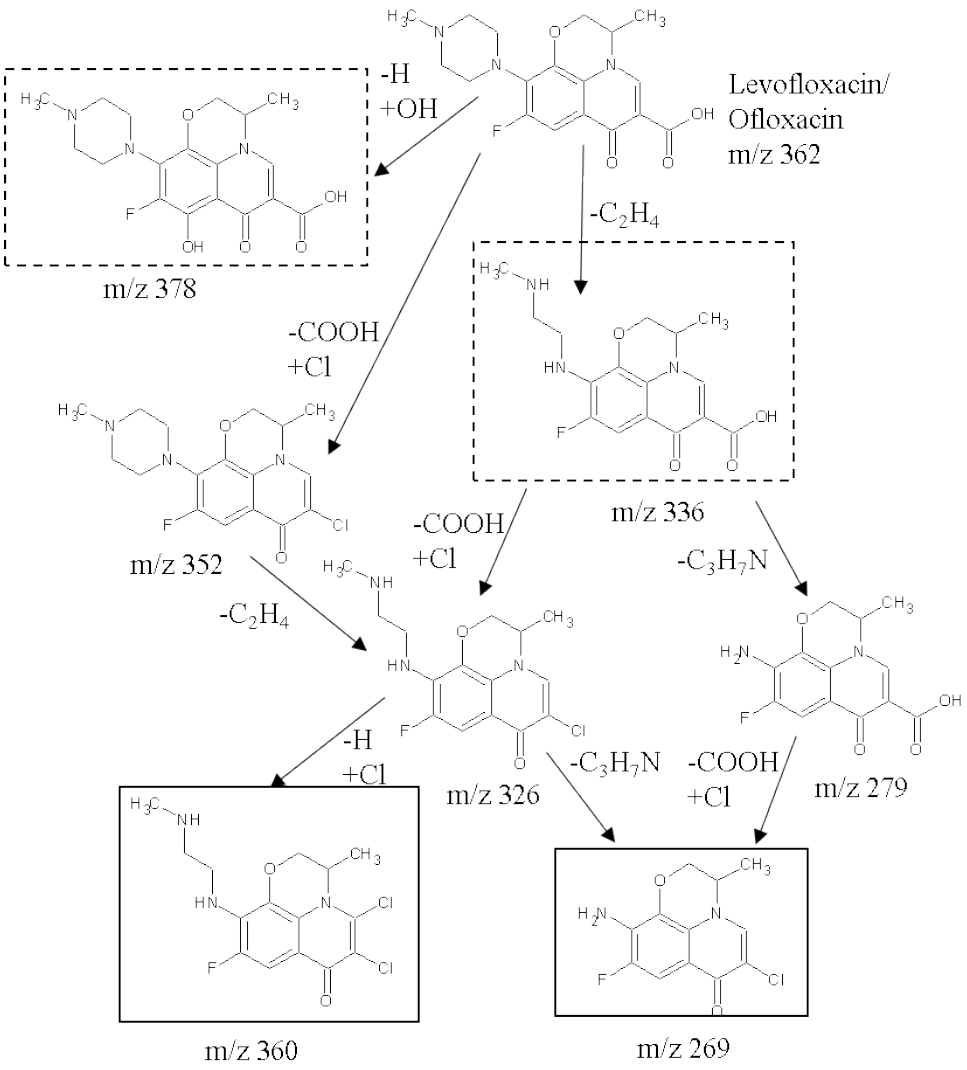
Levofloxacin and Ofloxacin:

Several chlorinated and non-chlorinated products of levofloxacin and ofloxacin formed (Figure 5). Wastewater matrix significantly affected the products that formed and their retention times. The following products were observed in wastewater matrix: *m/z* 326 (monochlorinated), *m/z* 352 (monochlorinated), *m/z* 336 (not chlorinated), *m/z* 378

(not chlorinated) and *m/z* 279 (not chlorinated). The products are listed in the order of increasing retention time with chlorinated products unexpectedly showing shorter retention times compared to non-chlorinated products. No major differences in products were observed for ofloxacin and levofloxacin. In ultrapure water, additional peaks were detected for *m/z* 269 (monochlorinated), *m/z* 360 (dichlorinated),and *m/z* 382 (dichlorinated), *m/z* 370 (monochlorinated). Products with m/z 382 and m/z 370 formed in low quantities and in ultrapure water only. Their structures were not investigated, as they appear to have little environmental relevance. For two of the products (*m/z* 326 and *m/z* 352), ofloxacin in ultrapure water showed symmetrical double peak indicating isomer formation (chlorine substitution at different locations), while levofloxacin showed only one peak for both of those products. This suggests that chirality has affected the reaction pathway. Both of these products had only a single peak in wastewater effluent matrix as well. It is also of note that products with *m/z* 336 and *m/z* 378 were not detected in ultrapure water. The labile locations on the molecule are the piperazinyl ring and the carboxyl group. Because of the competition for chlorine reactions from other substances in wastewater effluent matrix, both compounds were able to achieve slightly higher degree of transformation in ultrapure water. This explains why some of the more transformed products with a higher degree of chlorination were present in the ultrapure matrix, and not in wastewater effluent matrix. This also explains why an early intermediate product (*m/z* 336) was detected in the effluent matrix and not in ultrapure water matrix. The hydroxylated product (*m/z* 378) was likewise detected in the wastewater effluent matrix only. Hydroxylation is a common pathway in a reaction with free chlorine.[34] It is possible that this product was an intermediate that reacted further in

418 the ultrapure water matrix. The proposed location of the hydroxyl on the molecule is
419 based on susceptibility of aromatic rings to hydroxylation and on ortho-/para-directing
420 properties of fluorine. The proposed pathways are also supported by the fact that *m/z* 326
421 has 269 as the main fragment, *m/z* 336 has 279 as the main fragment, and *m/z* 378 has
422 362 (*m/z* of the parent compound) as the main fragment.

423 Of the detected products, *m/z* 352 and *m/z* 326 have been previously reported.[33,34]
424 A product with *m/z* 336 was previously reported as well, however a different structure
425 was reported in previous work for that product.[34] In general, partial loss of piperazine
426 ring and substitution of the carboxyl group with chlorine are two of the common reaction
427 pathways reported for levofloxacin and ofloxacin. Other pathways, such as elimination
428 of most of the piperazine ring, as in products *m/z* 279 and 269, were reported for these
429 compounds in other chemical reactions, such as photocatalysis and sonophotocatalysis,
430 suggesting that it is a labile location on the molecule.[39,40] Hydroxylation during chlorine
431 reaction was reported for ofloxacin in other studies, albeit at different locations on the
432 molecule in products of higher degree of transformation.[34]
433
434



435

436 **Figure 5:** Proposed transformation pathways for chlorination products of levofloxacin

437 and ofloxacin. Products in dashed-line boxes were detected in wastewater effluent matrix

438 only, while products in solid-line boxes were detected in ultrapure water only.

439

440 Chu and Fernandez (1989) discuss the effects of various substituents on quinolone

441 antibiotic activity.[41] The change in the nitrogen substituent on the quinolone structure

442 affects the activity of the given fluoroquinolone antibiotic against specific organism

443 types, making the molecule more effective against some organisms and less effective

against others. All of the products for the three fluoroquinolones investigated in this study retained that portion of the molecule unchanged. The combination of carboxylic acid and keto groups are essential for the DNA binding of fluoroquinolones. Any modifications of those groups have previously demonstrated the loss of activity.[41] This excludes the following products as potentially active: *m/z* 324 and 253 for ciprofloxacin, and *m/*z 269, 326, 352 and 360 for levofloxacin and ofloxacin. The fluorine is also part of the essential structure, which excludes *m/*z 288 for ciprofloxacin as one of the active products. Piperazinyl ring is important but less so. It has been shown to be a superior substituent on that position with respect to antibacterial activity. However, other substituents yield atibacterially active substances, although their activity can be moderate or weak, if the substituent is smaller. Substituents such as –H, -Cl and $–NH_2CH_2CH_2NH_2$, as well as some other linear substituents all demonstrated some degree of antibacterial activity.[41] Therefore, the likely products with antibacterial activity are those with *m/z* 306, 263, 297, 298 and 334 for ciprofloxacin, and 279, 336 and 378 for levofloxacin and ofloxacin. It must be noted that many of the active products still contain chlorine reactive moieties, such as amines and activated aromatic rings. At higher CT values, some of the likely active products may be transformed into compounds that do not have antibacterial activity.

Conclusions:

During chlorine disinfection of water, three antibiotics from fluoroquinolone class formed transformation products that retained antibacterial properties. Sulfamethoxazole and trimethoprim did not form antibacterially active products and did not appear to have

467 synergistic effects of the products. Several products were proposed for the antibiotics
468 with residual antibacterial activity, and those likely to have antibacterially active
469 properties were identified based on structure-activity relationships known for this class of
470 antibiotics. Further experimental work can confirm the products responsible for the
471 residual antibacterial activity observed. The structures are postulated based on m/z
472 values and literature references, and positive identification of active molecules will
473 require further analytical work.
474
475 Transformation products of pharmaceuticals forming in water and wastewater
476 treatment warrant a closer investigation, specifically those that can have human health or
477 ecotoxicological effects, such as antibiotics. The results of this study emphasize the need
478 for further evaluation of the presence of transformation products of antibiotics in
479 treatment works and their role in development of antibiotic resistance. Additionally, as
480 active products may be further transformed in disinfection where they no longer have
481 antibacterial activity, further research is necessary to identify the appropriate treatment
482 conditions to achieve this endpoint along with disinfection for microbial safety.
483

484 **Conflicts of interest**

485 There are no conflicts to declare.
486

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Acknowledgments:

The authors would like to thank the Department of Civil and Environmental Engineering new faculty start-up fund for the funding of this research project. Nicole Kennedy Neth was partially funded by Infrastructure and Environmental Systems PhD program GASP award. The LC/MS instrument was purchased under NSF MRI grant No. 1337873.

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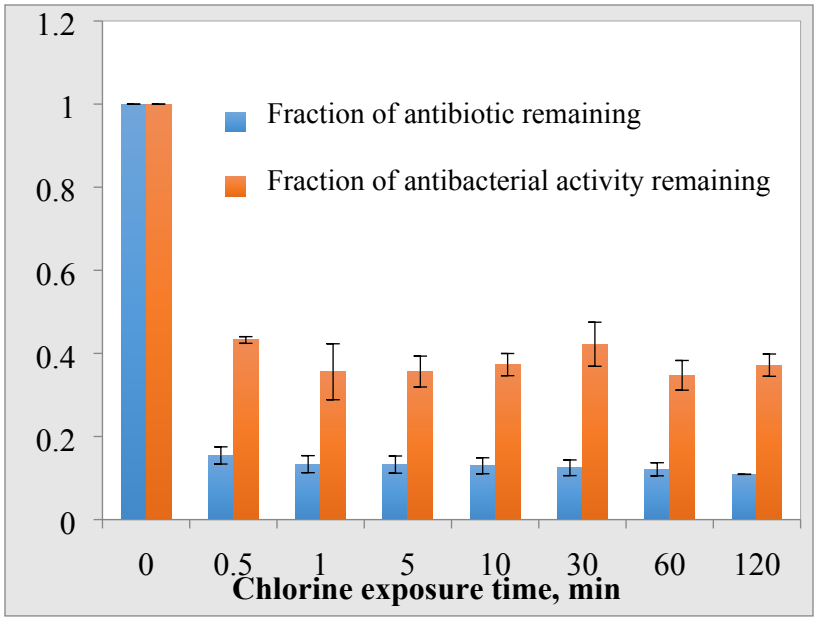
1.2
1
0.8
0.6
0.4
0.2
0
Fraction of antibiotic remaining
Fraction of antibacterial activity remaining
0
0.5
1
5
10
30
60
120
Chlorine exposure time, min