Environmental Science & Technology

Treatment and Resource Recovery

Effect-based identification of hazardous antibiotic transformation products after water chlorination

Adrian Jaen-Gil, Maria Jose Jose Farre Olalla, Alexandre Sànchez-Melsió, Albert Serra-Compte, Damià Barceló, and Sara Rodriguez-Mozaz

Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.0c00944 • Publication Date (Web): 26 Jun 2020

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Effect-based identification of hazardous antibiotic transformation products after water chlorination

Adrián Jaén-Gil [a,b*], *María-José Farré* [a,b], *Alexandre Sànchez-Melsió* [a,b], *Albert Serra-Compte* [a,b], *Damià Barceló* [a,b,c], *Sara Rodríguez-Mozaz* [a,b*]

[a] Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain

[b] Universitat de Girona, Girona, Spain

[c] Water and Soil Quality Research Group, Department of Environmental Chemistry, (IDAEA-CSIC), Jordi Girona 18-26, E-08034 Barcelona, Spain

KEYWORDS: Effect-directed analysis, suspect screening, antibiotic activity, acute toxicity.

ABSTRACT

Antibiotic transformation products (TPs) generated during water treatment can be considered of environmental concern, since they can retain part of the bioactivity of the parent compound. Effect-directed analysis (EDA) was applied for the identification of bioactive intermediates of azithromycin (AZI) and ciprofloxacin (CFC) after water chlorination. Fractionation of samples allowed the identification of bioactive intermediates by measuring the antibiotic activity and acute toxicity, combined with an automated suspect screening approach for chemical analysis.

25 While the removal of AZI was in line with the decrease of bioactivity in chlorinated
26 samples, an increase of bioactivity after complete removal of CFC was observed (at
27 > 0.5 mgCl_2/L). Principal component analysis (PCA) revealed that some of the CFC
28 intermediates could contribute to the overall toxicity of the chlorinated samples.
29 Fractionation of bioactive samples identified that the chlorinated TP296 (generated
30 from the destruction of the CFC piperazine ring) maintained 41%, 44% and 30% of
31 the antibiotic activity of the parent compound in chlorinated samples at 2.0, 3.0 and
32 4.0 mgCl_2/L, respectively. These results indicate the spectrum of antibacterial activity
33 can be altered by controlling the chemical substituents and configuration
34 of CFC structure with chlorine. On the other hand, the potential presence of volatile
35 DBPs and fractionation losses do not allow to tentatively confirm the main
36 intermediates contributing to the acute toxic effects measured in chlorinated
37 samples. Our results encourage further development of new and advanced
38 methodologies to study the bioactivity of isolated unknown TPs to understand their
39 hazardous effects in treated effluents.

1. INTRODUCTION

During the past decades, misuse and overuse of antibiotics have contributed to continuous discharges of these contaminants into the aquatic environment [1]. After human consumption, antibiotics are metabolised and excreted into sewage systems as pharmaceutically active forms [2,3]. While their administration clearly provides benefits for human health, the overuse of these substances in animal husbandry may also undergo serious potential risks [4,5]. In this sense, these substances are also widely applied for the treatment, prevention and prophylaxis in animals [6]. In most cases, conventional wastewater treatment plants (WWTPs) are not specifically designed for antibiotic removal and these contaminants are released into the receiving aquatic environment [7,8]. The presence of these pollutants in water bodies raises concern since they are associated with hazardous toxic effects and antibiotic resistance [1,9,10]. This is especially important in areas where treated effluents are used for water reuse activities and drinking water production [11]. Due to its low cost, good disinfection and oxidation capacity, chlorine (Cl_2) has been widely applied as a

post-treatment in WWTPs (and/or to maintain a residual chlorine in the distribution system) to protect public health by controlling microbial pathogens [12]. Even so, the presence of chlorine may lead to the formation of halogenated anthropogenic compounds and disinfection by-products (DBPs) which may have potential hazardous effects on the environment and to humans.

Among the antibiotics found in treated wastewater effluents, azithromycin (AZI) represented a breakthrough in the antibiotic era and became one of the best-selling branded antibiotics worldwide in 1980 [13,14]. Up to now, AZI was reported in raw urban wastewater up to 1 μg/L[7]. This antibiotic is characterised by its bioactivity against Gram-positive bacteria and greatly increase against Gram-negative bacteria to treat some respiratory tract and soft-tissue infections [13]. On the other hand, ciprofloxacin (CFC) has been the centre of considerable scientific interest since its discovery in the early 1980s [15]. Up to now, CFC was reported in raw urban wastewater up to 14 μg/L [7]. This pollutant represents one of the most common drugs in the treatment of bacterial infections from urinary tract, upper and low respiratory tract, skin, bone soft

tissue as well as pneumonia with increased potency against Gram-negative bacteria [16].

Although many efforts have been made to remove these antibiotic drugs from contaminated water, much less attention has been paid to the chlorinated and non-chlorinated intermediates generated after water disinfection. These unknown chemicals might retain part of the bioactivity of the parent compound and entail relevant concerns for the environment and public health even at low concentration levels [17]. In contact with aqueous chlorine, antibiotics may also undergo oxidation/substitution reactions yielding intermediates with high toxicity than their parent compounds [18]. Since reference standards are not commercially available for most of these unknown transformation products (TPs), the evaluation of their presence and hazardous effects cannot be performed. In this sense, effect-directed analysis (EDA) has overcome this challenge through the identification of bioactive chemicals in complex mixtures applying bioanalysis, separation and chemical analysis [19–22]. When potential effects are measured in collected samples, their

complexity is gradually reduced using fractionation liquid chromatography (LC) to further discard those fractions attaining low or absence of bioactivity [19]. In most of the cases, several fractionation steps are required until the isolated toxic fractions are ready for toxicant identification [19]. Final confirmation to assign their contribution effects is required using analytical approaches for structural identification, effect confirmation of artificial mixtures and hazard evaluation at different biological organisation levels [23].

For compound identification, many non-target and suspect screening methodologies have been developed for the identification of these substances by using databases or spectral information [24–27]. Since a broad variety of compounds (up to several thousands of features) can share a given molecular formula, the application of automated suspect screening methodologies by using prediction tools (such as prediction of TP exact masses, MS/MS fragmentation and retention times) may represent an important advance for rapid prioritisation of suspected chemicals present in samples [28,29]. Different commercial software and open-source programs

100 are available to optimise LC-MS data processing workflows for detection and
101 prioritisation of tentative chemical structures such as XCMS [30], enviMass [31], MZmine
102 2 [32], and Compound Discoverer [28]. Final confirmation of identified structures is
103 performed using e.g. reference standards and databases. In almost all cases, these
104 EDA approaches have been applied to real polluted waters but not for the
105 assessment of TPs. Only in a few cases, EDA has been applied to study the
106 bioactivity or ecotoxicity to unknown TPs generated in treatment processes when
107 references are not commercially available for confirmation [33–35].

108 In this study, an EDA methodology was developed for the identification and
109 elucidation of the bioactive TPs generated after AZI and CFC chlorination
110 experiments. The tentative TPs generated were isolated using a liquid-
111 chromatography system coupled to an automatic sample collector. The elucidation
112 of the generated intermediates was performed using a liquid-chromatography
113 system coupled to high-resolution mass spectrometry with an advanced and
114 automatic suspect screening methodology based on literature information and

compound prediction strategies. Antibacterial inhibition (i.e., antibiotic activity) and acute toxicity tests were employed to assess the ecotoxicological implications of the isolated unknown chemicals in chlorinated samples.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Azithromycin (AZI) and ciprofloxacin (CFC) were purchased at high purity grade (> 95%) from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water, acetonitrile and methanol LiChrosolv grade were supplied from Merck (Darmstadt, Germany). For antibiotic inhibition test, *Micrococcus luteus* ATCC 9341 and *Yersinia ruckeri* NCIMB 13282 were used in iso-sensitest agar (Oxid) and 2/3 Plate Count Agar (Difco) medium, respectively. *Vibrio fischeri* bacteria used for Microtox bioassay was purchased from Modern Water (Guildford, United Kingdom). Sodium hypochlorite solution (reagent grade, available chlorine ≥4%, Sigma-Aldrich) was used for the chlorination experiments. For all principal component analysis (PCA) calculations, the R Software version 3.5.3 was used.

130 *2.2 Experimental set-up*

131 Target pollutants (AZI and CFC) were spiked separately at an initial concentration
132 of 2.0 mg/L in ultra-pure water (buffered at pH 7.3 with sodium phosphate buffer (10
133 mM)) for a total working volume of 65 mL in triplicate experiments. Then, a proper
134 volume of chlorine (hypochlorite) was added to achieve the selected initial
135 concentrations of free available chlorine of 0.0, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 6.0
136 mgCl_2/L. In addition, a control experiment in ultra-pure water without spiking the
137 parent compounds was also performed. Batch flasks reactors were sealed avoiding
138 head space and introduced in an incubator at a constant temperature of 25 ºC. All
139 samples were collected after 24 h of treatment and the free available chlorine of
140 treated water was measured using commercial DPD (*N*, *N*-diethyl-p-
141 phenylenediamine) test kits (LCK310, Hach Lange) with a Hach DR2800
142 spectrophotometer (Düsseldorf, Germany). The final experimental time of 24h was
143 selected to maximise chlorination by-products formation while minimising hydrolysis
144 of the TPs. Experiments containing a concentration higher than 0.05 mgCl_2/L after

145 24 h were discarded for further analysis (actually only those samples with an initial
146 dose of 6.0 mgCl_2/L were finally discarded).

147 *2.3 Effect-directed analysis approach*

148 An adapted effect-directed analysis (EDA) methodology was applied for the
149 identification of the bioactive intermediates generated after water chlorination (Fig.
150 1) [36]. Briefly, samples collected after 24h of chlorination were biologically (antibiotic
151 activity and acute toxicity) and chemically (chromatographic and mass spectrometry
152 analysis) analysed. Then, computational assessment was performed for
153 identification of the chemicals present in samples. The bioactivity of each chlorinated
154 sample was plotted together with the presence of each identified intermediate
155 (chromatographic area of the TPs identified divided by the area of the
156 chromatographic peak of the parent compound at initial time) by principal component
157 analysis (PCA). With this information, the suspect hazardous intermediates present
158 in chlorinated samples were tentatively pointed out and the most representative
159 bioactive sample was selected for fractionation and further isolation of the TPs.

160 Sample fractions were again biologically and chemically analysed as well as
161 computationally assessed in duplicate to unravel their contribution as hazardous
162 chemicals in chlorinated treated samples.

163 *2.3.1 Biological analysis*

164 The antibiotic activity and acute toxicity endpoints were selected to evaluate the
165 hazardous effects of the antibiotics selected and TPs in chlorinated samples and
166 fractions. The antibiotic activity was chosen since it is related to the specific mode of
167 action (MoA) of these pollutants. In parallel, the acute toxicity was selected since it
168 is classified as a conventional endpoint measured in the environment.

169 To evaluate the antibiotic activity [37], the iso-sensitest agar (Oxid) medium with an
170 addition of 7.5 µg/L of tylosine (adjusted to pH 8.0) and inoculated with *M. luteus*
171 ATCC 9341 bacteria was used for samples collected from AZI experiments. In the
172 case of CFC experiments, 2/3 Plate Count Agar (Difco) 5% of 1M phosphate buffer
173 with an addition of 8,000 µg/L of cloxacilline (adjusted to pH 6.5) and inoculated with
174 *Y. ruckeri* NCIMB 13282 bacteria was used. In both cases, 35 mL of the inoculated

175 agar was poured into a 120 x 120 mm bioassay plate containing 9 holes per plate.
176 A volume of 250 µL of samples was transferred to individual holes with the addition
177 of 50 µL of 1M phosphate buffer. Then, sample plates were incubated at 30 ˚C for
178 16 hours. Antibiotic activity of samples was determined by observing the growth
179 inhibition of the bacterial culture and measuring the diameter of the non-bacterial cell
180 density corresponding to the absence of bacterial growth. All the values were
181 calculated related to the antibiotic activity of the parent compound before
182 chlorination.

183 Additionally, the ISO 11348-3 protocol for testing bacterial bioluminescence of
184 wastewater matrices was used to assess acute toxicity throughout Microtox® Model
185 500 Toxicity Analyser (Strategic Diagnostics Inc., Newark, DE, US) [38]. Stain of
186 luminescent bacteria *Vibrio fischeri* NRRL B-11177 were prepared from
187 commercially available freeze-dried reagents stored at -20 ˚C. A volume of 2 mL was
188 required for sample analysis. Then, the percentage of decay on emitted light was
189 measured when samples were in contact 15 min with the bioluminescent bacterium

190 *V. fischeri*. The data expressed as EC_{50} was transformed into toxicity units (TU =
191 $100/EC_{50}$) [39], where a higher TU indicates a greater effect [40].

192 2.3.2 *Chemical analysis*

193 A liquid chromatography system coupled to a high-resolution mass spectrometer
194 LC-LTQ-Orbitrap-MS/MS was used as described previously [41]. Briefly, 20 µL of
195 samples were injected and separated in a ZORBAX Eclipse XDB-C18 (150 mm ×
196 4.6 mm, 5 µm; Agilent Technologies, Santa Clara, CA). The mobile phases were (A)
197 10 mM ammonium formate in water at pH 3.0 and (B) acetonitrile. The optimised
198 chromatographic gradient was performed as follows [41]: initial mobile phase
199 composition (95% A) held for 1 min, followed by a decrease in composition A to 5%
200 within 9 min, then to 0% in 3 min, held for 2 min, and up to 95% in 1 min and held
201 for 1 min.

202 The high-resolution mass spectrometer LTQ-OrbitrapVelos™ (Thermo Fisher
203 Scientific) was equipped with a heated electrospray ionisation source (HESI-II). The
204 analysis was performed in positive and negative ionisation modes. As no peaks

attributed to TPs were found in negative ion mode chromatograms, further data processing was carried out only with that acquired in positive ion mode. Samples were acquired in full scan data acquisition from *m/z* 100 to 1,000 range at a resolving power of 60,000 FWHM. For structural elucidation of TPs, MS/MS fragmentation was performed in data dependent acquisition mode (DDA) at 30,000 FWHM from *m/z* 100 to 1,000 range, for the three most intense ions from a selected list of 16 exact masses corresponding to potential AZI (Table S1) and 13 exact masses for CFC collected from literature (Table S2), (pre-acquisition suspect screening approach). If selected masses were not found, the three most intense ions detected in a full-scan MS spectra were automatically selected for fragmentation. All data were further processed with a post-acquisition suspect screening approach (Section 2.3.3). Additionally, isotopic data-dependent (IDD) was performed for the expected isotopic ratios of 0.32 and 0.64 comprising a mass difference of 1.9971 Da. All MS/MS experiments were performed applying a dynamic mass exclusion mode to discriminate co-eluted compounds: ions fragmented more than three times during

220 25 seconds were further ignored for fragmentation during the following 30 seconds
221 (corresponding to peak plus tailing). Mass spectrometry conditions were designed
222 as follows: spray voltage, 3.5 kV; source heated at 300 ˚C; capillary temperature,
223 350 ˚C; sheath gas flow, 40 (arbitrary units); and auxiliary gas flow; 20 (arbitrary
224 units) [41]. Fragmentation techniques selected were: collision-induced dissociation
225 (CID) at a normalised collision energy of 30 eV (activation Q of 0.250 and an
226 activation time of 30 ms) and higher-energy collisional dissociation (HCD) at a
227 normalised collision energy of 55 eV (activation time of 0.100 ms) with isolation width
228 of 2 Da. The entire system was controlled via Aria software under Xcalibur 2.1.

229 *2.3.3 Computational analysis*

230 An advanced post-acquisition suspect screening approach for identification of the
231 TPs generated in chlorination experiments and collected in the corresponding
232 fractions was applied using Compound Discoverer 3.0 (Thermo Fisher Scientific Inc.,
233 Waltham, MA). The adapted methodology is presented in Fig. S1 and Table S3 [42].
234 Briefly, input files containing the chromatograms and mass spectra files from

analysed samples were loaded separately into the software. In addition, chemical structures of AZI and CFC were also loaded to further create a list of tentative TPs predicted by the software after applying the following chemical reactions to the parent compound structures (a maximum combination of three): dehydration, desaturation, reduction, oxidative deamination to ketone, oxidative deamination to alcohol, chlorination, hydration, oxidation, reductive defluorination and dealkylation. A number of 1655 and 497 exact masses were predicted from AZI and CFC chemical structures, respectively. Automatic data processing starts with filtering MS data between 100 and 1000 Da and from 1 to 12 min with a S/N ratio of 3. To compensate for small differences in retention times, chromatographic alignment was performed by using a mass tolerance error of ±5 ppm and a maximum retention time shift of 0.3 min. Immediately after, data processing was performed by searching the predicted list of TP exact masses in sample files. Then, the fragments present in collected MS/MS data were automatically matched with the predicted fragments generated using *in silico* fragmentation with a mass tolerance error of ± 5 ppm. Those

250 compounds with FISh (Fragment Ion Search) coverages higher than 65% were
251 selected for data evaluation [42].

252 *2.3.4 Statistical analysis for the estimation of hazardous TPs*

253 Additionally, principal component analysis (PCA) was used to evaluate the
254 correlations between the bioactivity measured (antibiotic activity and acute toxicity,
255 separately) and the TPs identified in chlorinated samples, following the approach
256 previously reported [43]. Relative areas in percentage values (area of the peaks
257 detected in chromatogram divided by the area of the chromatographic peak of CFC
258 before the treatment) were used as input value using the FactoMineR included in
259 the Rcmdr environment (RcmdrPlugin.FactoMineR interface) for automatic data
260 processing. For all calculations, the R Software version 3.5.3 was used.

261 *2.3.5 Sample fractionation*

262 Taking into account the PCA results of the suspected bioactive intermediates
263 present in chlorinated samples, the most representative bioactive sample containing
264 all the TPs identified was selected for sample fractionation (2 mgCl_2/L vs 2 mg/L of

265 CFC at an initial time). To achieve a proper concentration of the isolated TPs in one
266 fractionation cycle, this experiment was repeated at a higher concentration adding
267 the corresponding proportion of reactants (10 mgCl_2/L vs 10 mg/L CFC at an initial
268 time). This is considered a critical step since a minimal concentration of the TPs
269 (normally present at low concentration levels) is required to further reach detection
270 limits during LC-MS/MS analysis and the selected bioassays. Then, fractionation
271 was performed by using a preparative HPLC Agilent 1260 Infinity high-pressure
272 liquid-chromatography system coupled to a diode array detector (HPLC-DAD). The
273 fraction collection was automatically carried out in a 1100/1200 fraction collector
274 G1364C using a diverter valve to switch from waste to the collector position. A
275 volume of 100 µL of samples was injected in a ZORBAX Eclipse XDB-C18 column
276 (150 mm × 4.6 mm, 5 µm; Agilent Technologies, Santa Clara, CA) at a flow rate of 1
277 mL/min and column temperature of 25˚C. Pure mobile phases selected were (A)
278 pure water and (B) acetonitrile to avoid the presence of any residual interference
279 affecting to bioassay measurements. The chromatographic gradient was carried out

as follows: initial mobile phase composition (90% A) held for 5 min; to 40% in 1 min and held for 7 min; to 30% in 1 min and held for 6 min; to 20% in 1 min and held for 6 min; to 10% in 1 min and held for 6 min; and to 90% in 1 min held for 5 min. Detection was monitored at the maximum absorption wavelength of 271 nm measured in a UV-1800 UV-VIS spectrophotometer (Shimadzu Inc., Kyoto, Japan). The total volume collected for each fraction was approximately 2 mL. Since reference standards of TPs are not available to quantify recoveries in solid-phase extraction and pharmaceutical TPs are not usually volatile, fractions were collected in glass collectors, evaporated to dryness with nitrogen and reconstituted in 0.5 mL of pure water. Reconstitution in pure water was carried out to prevent the presence of organic solvents interfering on bacteria integrity on the bioanalysis of fractions [44–46]. In addition, since pure water was the solvent used in chlorination experiments, a better comparison with the fractions collected is assured. All fractions collected were evaluated using biological (by antibiotic activity and acute toxicity measurement as explained in biological analysis section) and chemical (using the LTQ-Orbitrap for

295 MS/MS compound identification, as explained in chemical analysis section)
296 analyses. Finally, computational assessment (using Compound Discoverer, as
297 presented in the computational analysis section) was performed for compound
298 identification.

299 3. RESULTS AND DISCUSSION

300 *3.1 Biological and chemical analyses of chlorinated samples*

301 Chemical analyses revealed an AZI removal up to 88% after 24h of the addition of
302 the highest chlorine dose (4 mgCl_2/L) (Fig. 2a). This percentage of elimination is in
303 line with the decrease in the initial antibiotic activity up to 85% (Fig. 2b). As observed
304 in other oxidation treatment processes reported in the literature [47], the decrease on
305 the measured antibiotic activity is due to the elimination of the parent compound as
306 none of the intermediates generated had any relevant contribution to the overall
307 antibiotic activity. In terms of acute toxicity, no effects were observed after any of the
308 chlorination experiments performed (Fig. 2c). These results are in accordance with
309 those reported in the literature about oxidation treatment processes, where the

absence or reduction of the toxicity of the intermediates generated after AZI degradation was observed [48,49].

On the other hand, chlorination promoted complete CFC elimination (*ca.*100%) after 24h of treatment adding an initial chlorine dose of 0.5 mgCl_2/L (Fig. 2a). These results are in line with the negligible antibiotic activity at 0.5 mgCl_2/L (Fig. 2b). The antibiotic activity exceeded 41%, 44%, and 30% of the initial effect of the parent compound in the experiments performed at 2, 3, and 4 mgCl_2/L, respectively (Fig. 2b). This fact suggests that some of the CFC intermediates generated might retain part of the antibiotic activity of the parent compound. Controversial data about the antibiotic activity of the intermediates generated during CFC degradation have been reported in the literature. A reduction on antibacterial activity regarding the elimination of CFC was generally observed after photolytic, photocatalytic, electrochemical and Fe (VI) oxidation [50–54]. In some cases, it was reported a negligible antibacterial potency of the TPs generated in those water treatments [54]. On the contrary, the bioactivity of the intermediates generated was sometimes

325 detected after ozonation treatment [55], in line with the results obtained in this study in
326 chlorination experiments.

327 Negligible acute toxicity was measured in the absence of chlorine in CFC
328 experiments (Fig. 2c). The maximum increase up to 4.1 TU was observed after
329 adding 2 mgCl_2/L (Fig. 2c). Also, in this case, controversial data about acute toxicity
330 of the intermediates generated during CFC degradation have been reported in the
331 literature. For instance, a decrease in acute toxicity was observed after sonolysis
332 and UV treatment experiments spiked at 15 mg/L of CFC [56,57]. On the contrary, an
333 increase in acute toxicity of about 18% was observed from the TPs generated after
334 1 hour of CFC chlorination at 10 molar equivalents of chlorine dose [58], and up to
335 26% after radiation-induced experiments at 33 mg/L of CFC [59].

336 As shown in this study, the potential intermediates generated during chlorination
337 of AZI did not show any effect in the biological tests applied. The intermediates
338 generated during CFC experiments were pinpointed as concerning TPs since acute
339 toxicity and antibiotic activity were measured after chlorination experiments.

340 *3.2 Computational analysis of chlorinated samples and elucidation of*
341 *transformation pathways*

342 Thirteen TPs were tentatively identified in AZI chlorinated samples (Table S4), and
343 the transformation pathway and their relative presence are presented in Fig. 3. As
344 previously reported, the elimination of the parent compound was mainly led by O-
345 dealkylation of L-cladinose moiety (TP590) suggested from the instability of
346 macrolides in aqueous solution [42], and confirmed by its presence at the initial time
347 up to 80%. However, the increase of initial chlorine concentration led to its further
348 elimination reaching a presence of 12% in treated samples when 4 mgCl_2/L was
349 added at the initial time. This intermediate was mainly transformed into TP576 after
350 demethylation of the dimethylamine group in D-desosamine moiety [42]. As reported
351 previously [60], both hydrolysis of D-desosamine moiety and demethylation were also
352 observed directly from AZI parent compound being transformed into TP591 and
353 TP734A (and found in this study up to 132% and 65% at 4 mgCl_2/L, respectively). It
354 is important to mention that none of the most intense compounds elucidated contains

355 a chlorine substituent in their chemical structures after the experiments performed.
356 As previously reported [60], the pseudo-first order kinetic constants at different pH
357 values showed that the reactivity of AZI with free available chlorine was favoured at
358 higher pH within the range of 7.5 and 8.5 (optimal pH value was 8.0). Therefore, the
359 use of a pure water pH at 7.3 may explain the low presence of halogenated TPs after
360 chlorination experiments. As explained previously and in Fig. 2, none of these
361 elucidated intermediate structures from AZI presented hazardous effects in treated
362 effluents.

363 Seven TPs were tentatively identified in CFC chlorinated samples (Table S5). The
364 transformation pathway and their relative presence are presented in Fig. 4. As
365 previously reported [61], initial chlorination of CFC structure induced the destruction of
366 the piperazine ring moiety into TP365 (not detected in this study). The instability of
367 TP365 probably led to the opening of the piperazine ring and rapidly transformed
368 (though imine hydrolysis and the loss of CH_2O) into TP305 [61–63], which was detected
369 in this study at high percentage values (32% relative area to the initial area of CFC)

370 when 0.5 mgCl_2/L of chlorine was added. However, the increase of initial chlorine
371 concentration led to its further elimination reaching low levels (4%) at 2 mgCl_2/L of
372 chlorine dose. In fact, TP305 was most likely transformed by *N*-chlorination, and
373 further elimination of the $C_2H_3NCl_2$ moiety generating the compound TP262. In
374 comparison to TP305, TP262 attained the highest concentration when chlorine was
375 added at 2.0 mgCl_2/L at an initial time (up to 95% from the initial presence of CFC).
376 These results are in accordance with the previous data reported in the literature
377 where TP262 formation was observed from 12.4% (after 2h of chlorination treatment)
378 to 54.4% (after 50h) [61]. Additionally, TP262 was also found as the main intermediate
379 generated in photo-Fenton degradation experiments after 30 min of treatment, 1.5
380 times-fold higher when compared with the other generated TPs [51]. Finally, the
381 presence of additional intermediates in samples with an excess of chlorine indicates
382 that TP305 may generate further chlorinated compounds in treated samples: the
383 chlorinated TP296 was observed up to 39% when decreasing the presence of TP262
384 to 5% at an initial chlorine concentration of 4.0 mgCl_2/L. This fact indicated that the

385 highest extent on transformation pathway was achieved when increasing the
386 chlorine concentration [62]. The most significant concentration of TP296 was detected
387 at 3.0 mgCl_2/L of chlorine up to 44%. Otherwise, other oxidation intermediates (e.g.
388 TP333) were also identified from CFC [61] at a lower concentration than 10% after
389 chlorination experiments (Fig. 4). As explained previously and in Fig. 2, some of the
390 elucidated intermediate structures for CFC presented hazardous effects in treated
391 effluents.

392 *3.3 Estimation of the hazardous TPs generated in CFC chlorinated samples*

393 Estimation of the tentative hazardous TPs identified in CFC experiments (in terms
394 of antibiotic activity and acute toxicity) was evaluated using PCA plots (Fig. 5), which
395 allowed correlating bioactivity measured with the presence of individual TPs
396 identified in chlorinated samples (Table S6 and Table S7). Since variables were
397 measured on different scales (relative percentages and toxic units), both PCAs were
398 normalised to the specific range of [-1, +1]. The direct correlation of an intermediate
399 with a given effect estimates its tentative hazardous contribution in chlorinated

400 samples. The two first principal components (PCs) pointed out the TP296 (Fig. 5a)
401 as the key intermediate contributing to the increase of 41%, 44%, and 30% in the
402 antibiotic activity in treated samples of the experiments performed at 2, 3, and 4
403 $mgCl_2/L$, respectively (Fig. 2b). This may be due to the different chemical
404 substituents of this intermediate which may govern antibacterial efficacy and
405 influence the side-effect profile [64]. On the contrary, an inverse correlation was
406 observed for TP339, TP305 and TP333. Otherwise, PCA loadings pointed out a
407 direct correlation between acute toxicity of chlorinated samples and the presence of
408 TP262, TP290 and TP292 (Fig. 5b). Since TP262 was found in chlorinated samples
409 at the major relative presence of 95% at 2.0 $mgCl_2/L$ (Fig. 4), it might be classified
410 as the key intermediate contributing to the increase of 4.1 TU in the acute toxicity in
411 chlorinated samples (Fig. 2c). The contribution of TP290 and TP292 may also exhibit
412 higher toxicity of these intermediates compared to the rest of TPs, and therefore,
413 despite their apparently low relative presence (Fig. 4), they may also contribute to
414 the total toxicity of the samples. In addition, the synergic effects of these compounds

415 cannot be discarded. Otherwise, an inverse correlation was observed for TP339 and
416 TP296. These intermediates may result in lower steric resistance and easier
417 penetration into a cell of luminescent bacteria [65], which subsequently might lead to
418 an increase in toxicity. Additionally, as expected the electronegative atoms
419 contained in quinolone molecules (such as F, N, and O atoms) may donate electrons
420 to photo-bacterium and thus inhibited the luminance emission [58].

421 In both statistical experiments (Fig. 5a and Fig. 5b), the generation of low
422 molecular mass DBPs, not considered in this study, might have also contributed to
423 the hazardous effects measured in treated samples. In this context, it was previously
424 reported that monochloroacetic acid was the main DBP formed during chlorination
425 of CFC and detected at a concentration around 100 μg/L after 24 hours (when CFC
426 was spiked at 16 mg/L adding a chlorine dose of 1 mM) [66]. In our conditions selected,
427 the formation of monochloracetic acid was expected to be minimal since CFC was
428 spiked at a much lower concentration of CFC (2 mg/L) and chlorine dose (0.056
429 mM). Taking into account the PCA results, which tentatively pointed out TP296 and

TP262 as hazardous intermediates, fractionation and further chemical and biological analyses, as well as computational assessment, were needed to confirm or rule out the risk of these intermediates in chlorinated samples.

3.4 Confirmation of the hazardous TPs generated in chlorinated samples

Taking into account the PCA results, the most representative sample containing all the TPs identified was selected for sample fractionation (2 mgCl_2/L vs 2 mg/L of CFC at an initial time). To achieve a proper concentration of the isolated TPs without launching several sample fractionation cycles, the experiment was repeated at a higher concentration adding the same proportion of reactants (10 mgCl_2/L vs 10 mg/L CFC at initial time). After sample fractionation (Fig. 6a), measurements on antibiotic activity and acute toxicity indicated no toxic effects (in comparison to control samples) when no chromatographic peaks were detected in the fractions collected (every 2 min approximately) at the beginning and the end of the chromatogram (Fig. 6b). Chromatographic and mass spectra data from LC-MS/MS (Orbitrap Velos^{TM}) system of fractions showed that TP333 was found in fraction 2

445 but at low concentration values (Fig. 6c). Two intense peaks in fractions 3 and 4
446 were assigned to TP262 (generated after the elimination of the piperazine ring
447 moiety), and TP296 (a further chlorinated intermediate of the transformation
448 pathway), respectively (Fig. 4). Conversely, no compound assignment was possible
449 for fraction 5 (Fig. 6c).

450 Biological analysis showed a relative antibiotic activity of around 1% in fraction 2,
451 4% in fraction 3, 52% in fraction 4, and 3% in fraction 5 (Fig. 6b). The significant
452 presence of TP296 in fraction 4 was in agreement with the 52% of antibiotic activity
453 measured in this fraction. For this intermediate, the fractionation process allowed to
454 recover the 78% of the chromatographic area from the initial chlorinated sample.
455 Therefore, TP296 (the chlorinated molecule generated from TP305, Fig. 4) was
456 tentatively identified to retain the antibiotic activity of its parent compound CFC in
457 chlorinated samples. Nonetheless, despite 22% of compound losses were observed
458 during fractionation, synergistic and antagonistic effects cannot be discarded. These
459 results are in accordance with the reported literature indicating that the spectrum of

460 antibacterial activity can be altered by controlling the substitution and configuration
461 of position 8 on CFC structure with C-F, C-Cl and N substituents (Fig. 6a), and
462 expanding the antibacterial spectrum against anaerobes [15,64,67,68]. Additionally, these
463 results confirm the suitability of PCA estimations to identify the most hazardous
464 intermediates generated during water treatment in terms of antibiotic activity (Fig. 5).
465 On the other hand, while chlorinated samples were toxic at an initial concentration
466 of 2 mgCl_2/L (Fig 2c), no acute toxicity was observed in any of the fractions collected
467 exceeding EC_{50} values. In this context, calculations of EC_{10} were performed from the
468 slope of the linear regression of concentration vs. % effect and converted in TU
469 values as reported previously [69,70] (Fig. S3). In particular, the most abundant
470 intermediate highlighted by chlorination experiments TP262 (Fig. 4) was found at the
471 highest acute toxic value of 4.6 TU in fractions collected (Fig. 6b and Fig. 6c). Yet,
472 the fractionation process allowed to recover only 42% of the TP based on the
473 chromatographic areas before and after fractionation. Therefore, partial loss of this
474 TP along sample evaporation and fractionation might also contribute to the reduction

475 of acute toxicity measured in the fractions. It is important to mention that other
476 estimated toxic intermediates (such as TP290 and TP292), present at low
477 concentration levels were also affected by fractionation losses since they were not
478 detected in fractions collected.

479 The identification of the most relevant intermediates in terms of antibiotic activity
480 generated from chlorination experiments with CFC was successfully achieved using
481 an EDA approach, which includes fractionation of bioactive samples in combination
482 with biological, chemical and computational assessment using an automated
483 suspect screening methodology. The TP296 (generated from the destruction of the
484 piperazine ring moiety and its further chlorination) was identified to maintain 41%,
485 44% and 30% of the antibiotic activity of the parent compound in chlorinated samples
486 at 2.0, 3.0 and 4.0 mgCl_2/L, respectively (Fig. 2) being classified as a potentially
487 concerning intermediate after water chlorination. Therefore, the use of EDA
488 approaches in combination with PCA evaluation represents a potential approach for
489 the identification and confirmation of hazardous TPs in treated samples. Although

490 the complete elimination of antibiotics should eventually be the objective of water
491 treatment processes, the elimination of the potential bioactive TPs generated is also
492 required, even when complete elimination of the parent compound is attained.

493 ## ASSOCIATED CONTENT

494 **Supporting Information**. The post-acquisition data processing workflow and
495 parameters selected, the exact mass list of the most common azithromycin and
496 ciprofloxacin TPs found in literature, the detected and identified a list of AZI and CFC
497 TPs in chlorination experiment, the UV spectra of ciprofloxacin intermediates
498 identified, the R-Scripts and acute toxicity measurements of fractions are provided
499 in the Supporting Information.

500 ## AUTHOR INFORMATION

501 ### Corresponding Authors

*Sara Rodriguez-Mozaz (srodriquez@icra.cat), Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain, Tel: (+34) 972 18 33 80

*Adrián Jaén-Gil (ajaen@icra.cat), Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain, Tel: (+34) 972 18 33 80

Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGEMENTS

This work was funded by the Spanish State Research Agency of the Spanish Ministry of Science, Innovation and Universities AEI-MICIU and the Fondo Europeo de Desarrollo Regional under the National Program for Research Aimed at the Challenges of Society (CTM2017-85335-R). Authors acknowledge the support from the Economy and Knowledge Department of the Catalan Government through the

Consolidated Research Group (ICRA-ENV 2017 SGR 1124 and 2017-SGR-1404). ICRA researchers thank funding from CERCA program. M.J.F and S.R.M. acknowledge the Ramon y Cajal research fellowships RyC-2015-17108 and RYC-2014-16707 from the Spanish Ministry of Economy and Competitiveness. A.J.G. and A.S.C acknowledges the predoctoral grants 2019FI_B2_00202 and 2018FI_B2_00170 from AGAUR and co-financed by the European Social Fund. The authors would like to Wageningen Food Safety Research for their help with the antibiotic inhibition tests.

REFERENCES

(1) Rodriguez-Mozaz, S.; Chamorro, S.; Marti, E.; Huerta, B.; Gros, M.; Sànchez-Melsió, A.; Borrego, C. M.; Barceló, D.; Balcázar, J. L. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* **2015**, *69*, 234–242.

(2) Yang, Y.; Ok, Y. S.; Kim, K. H.; Kwon, E. E.; Tsang, Y. F. Occurrences and

530 removal of pharmaceuticals and personal care products (PPCPs) in drinking
531 water and water/sewage treatment plants: A review. *Sci. Total Environ.* **2017**,
532 *596–597*, 303–320.

533 (3) aus der Beek, T.; Weber, F. A.; Bergmann, A.; Hickmann, S.; Ebert, I.; Hein,
534 A.; Küster, A. Pharmaceuticals in the environment-Global occurrences and
535 perspectives. *Environ. Toxicol. Chem.* **2016**, *35* (4), 823–835.

536 (4) Lees, K.; Fitzsimons, M.; Snape, J.; Tappin, A.; Comber, S. Pharmaceuticals
537 in soils of lower income countries: Physico-chemical fate and risks from
538 wastewater irrigation. *Environ. Int.* **2016**, *94*, 712–723.

539 (5) Durso, L. M.; Cook, K. L. Impacts of antibiotic use in agriculture: What are the
540 benefits and risks? *Curr. Opin. Microbiol.* **2014**, *19* (1), 37–44.

541 (6) Wegener, H. C. Antibiotics in animal feed and their role in resistance
542 development. *Curr. Opin. Microbiol.* **2003**, *6* (5), 439–445.

543 (7) Verlicchi, P.; Al Aukidy, M.; Zambello, E. Occurrence of pharmaceutical

544 compounds in urban wastewater: Removal, mass load and environmental risk
545 after a secondary treatment-A review. *Sci. Total Environ.* **2012**, *429*, 123–155.

546 (8) Grenni, P.; Ancona, V.; Barra Caracciolo, A. Ecological effects of antibiotics
547 on natural ecosystems: A review. *Microchem. J.* **2018**, *136*, 25–39.

548 (9) Martinez, J. L. Environmental pollution by antibiotics and by antibiotic
549 resistance determinants. *Environ. Pollut.* **2009**, *157* (11), 2893–2902.

550 (10) Chartier, Y.; Emmanuel, J.; Pieper, U.; Prüss, A.; Rushbrook, P.; Stringer, R.;
551 Townend, W.; Wilburn, S.; Zghondi, R. *Safe Management of Wastes from*
552 *Health-Care Activities*; World Heal. Organ.: Geneva Switz, 2014.

553 (11) Huerta-Fontela, M.; Galceran, M. T.; Ventura, F. Occurrence and removal of
554 pharmaceuticals and hormones through drinking water treatment. *Water Res.*
555 **2011**, *45* (3), 1432–1442.

556 (12) Diana, M.; Felipe-Sotelo, M.; Bond, T. Disinfection byproducts potentially
557 responsible for the association between chlorinated drinking water and

558 bladder cancer: A review. *Water Res.* **2019**, *162*, 492–504.

559 (13) Jeli, D.; Antolovic, R. From Erythromycin to Azithromycin and New Potential

560 Ribosome-Binding Antimicrobials. *Antibiotics* **2016**, *5* (29), 1–13.

561 (14) Kagkelaris, K. A.; Makri, O. E.; Georgakopoulos, C. D.; Panayiotakopoulos, G.

562 D. An eye for azithromycin: review of the literature. *Ther. Adv. Ophthalmol.*

563 **2018**, *10*, 251584141878362.

564 (15) Andersson, M. I.; Macgowan, A. P. Development of the quinolones. *J.*

565 *Antimicrob. Chemother.* **2003**, *51*, 1–11.

566 (16) Ezelarab, H. A. A.; Abbas, S. H.; Hassan, H. A.; Abuo-Rahma, G. E.-D. A.

567 Recent updates of fluoroquinolones as antibacterial agents. *Arch. der*

568 *Parmazie* **2018**, No. May, 1–13.

569 (17) Escher, B. I.; Fenner, K. Recent Advances in Environmental Risk Assessment

570 of Transformation Products. *Environ. Sci. Technol.* **2011**, *45* (9), 3835–3847.

571 (18) Postigo, C.; Richardson, S. D. Transformation of pharmaceuticals during
572 oxidation/disinfection processes in drinking water treatment. *Journal of*
573 *Hazardous Materials*. 2014, pp 461–475.

574 (19) Brack, W.; Ait-Aissa, S.; Burgess, R. M.; Busch, W.; Creusot, N.; Di Paolo, C.;
575 Escher, B. I.; Mark Hewitt, L.; Hilscherova, K.; Hollender, J.; Hollert, H.; Jonker,
576 W.; Kool, J.; Lamoree, M.; Muschket, M.; Neumann, S.; Rostkowski, P.;
577 Ruttkies, C.; Schollee, J.; Schymanski, E L.; Schulze, T.; Seiler, T B.; Tindall,
578 A J.; De Aragão Umbuzeiro, G.; Vrana, B.; Krauss, M. Effect-directed analysis
579 supporting monitoring of aquatic environments - An in-depth overview. *Sci.*
580 *Total Environ.* **2016**, *544*, 1073–1118.

581 (20) Brack, W. Effect-directed analysis: a promising tool for the identification of
582 organic toxicants in complex mixtures? *Anal. Bioanal. Chem.* **2003**, *377*, 397–
583 407.

584 (21) Schymanski, E. L.; Bataineh, M.; Goss, K. U.; Brack, W. Integrated analytical

585 and computer tools for structure elucidation in effect-directed analysis. *TrAC -*
586 *Trends Anal. Chem.* **2009**, *28* (5), 550–561.

587 (22) Escher, B. I.; Stapleton, H. M.; Schymanski, E. L. Tracking complex mixtures
588 of chemicals in our changing environment. *Science.* **2020**, *367*, 388–392.

589 (23) Brack, W.; Schmitt-Jansen, M.; MacHala, M.; Brix, R.; Barceló, D.;
590 Schymanski, E.; Streck, G.; Schulze, T. How to confirm identified toxicants in
591 effect-directed analysis. *Anal. Bioanal. Chem.* **2008**, *390* (8), 1959–1973.

592 (24) Bletsou, A. A.; Jeon, J.; Hollender, J.; Archontaki, E.; Thomaidis, N. S.
593 Targeted and non-targeted liquid chromatography-mass spectrometric
594 workflows for identification of transformation products of emerging pollutants
595 in the aquatic environment. *TrAC - Trends Anal. Chem.* **2015**, *66*, 32–44.

596 (25) Díaz, R.; Ibáñez, M.; Sancho, J. V.; Hernández, F. Target and non-target
597 screening strategies for organic contaminants, residues and illicit substances
598 in food, environmental and human biological samples by UHPLC-QTOF-MS.

599 *Anal. Methods* **2012**, *4* (1), 196–209.

600 (26) Li, Z.; Kaserzon, S. L.; Plassmann, M. M.; Sobek, A.; Gómez Ramos, M. J.;
601 Radke, M. A strategic screening approach to identify transformation products
602 of organic micropollutants formed in natural waters. *Environ. Sci. Process.*
603 *Impacts* **2017**, *19* (4), 488–498.

604 (27) Schymanski, E. L.; Singer, H. P.; Slobodnik, J.; Ipolyi, I. M.; Oswald, P.;
605 Krauss, M.; Schulze, T.; Haglund, P.; Letzel, T.; Grosse, S.; Thomaidis, N S.;
606 Bletsou, A.; Zwiener, C.; Ibáñez, M.; Portolés, T.; De Boer, R.; Reid, M J.;
607 Onghena, M.; Kunkel, U.; Schulz, W.; Guillon, A.; Noyon, N.; Leroy, G.; Bados,
608 P.; Bogialli, S.; Stipaničev, D.; Rostkowski, P.; Hollender, J. Non-target
609 screening with high-resolution mass spectrometry: Critical review using a
610 collaborative trial on water analysis. *Anal. Bioanal. Chem.* **2015**, *407* (21),
611 6237–6255.

612 (28) Avagyan, R.; Åberg, M.; Westerholm, R. Suspect screening of OH-PAHs and

613 non-target screening of other organic compounds in wood smoke particles
614 using HR-Orbitrap-MS. *Chemosphere* **2016**, *163*, 313–321.

615 (29) Gago-Ferrero, P.; Krettek, A.; Fischer, S.; Wiberg, K.; Ahrens, L. Suspect
616 Screening and Regulatory Databases: A Powerful Combination to Identify
617 Emerging Micropollutants. *Environ. Sci. Technol.* **2018**, *52* (12), 6881–6894.

618 (30) Smith, C. A.; Want, E. J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. XCMS:
619 Processing mass spectrometry data for metabolite profiling using nonlinear
620 peak alignment, matching, and identification. *Anal. Chem.* **2006**, *78* (3), 779–
621 787.

622 (31) Albergamo, V.; Schollée, J. E.; Schymanski, E. L.; Helmus, R.; Timmer, H.;
623 Hollender, J.; De Voogt, P. Nontarget screening reveals time trends of polar
624 micropollutants in a riverbank filtration system. *Environ. Sci. Technol.* **2019**,
625 *53* (13), 7584–7594.

626 (32) Pluskal, T.; Castillo, S.; Villar-Briones, A.; Orešič, M. MZmine 2: Modular

framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* **2010**, *11*.

(33) Schulze, T.; Weiss, S.; Schymanski, E.; von der Ohe, P. C.; Schmitt-Jansen, M.; Altenburger, R.; Streck, G.; Brack, W. Identification of a phytotoxic photo-transformation product of diclofenac using effect-directed analysis. *Environ. Pollut.* **2010**, *158* (5), 1461–1466.

(34) Brack, W.; Altenburger, R.; Küster, E.; Meissner, B.; Wenzel, K. D.; Schüürmann, G. Identification of toxic products of anthracene photomodification in simulated sunlight. *Environ. Toxicol. Chem.* **2003**, *22* (10), 2228–2237.

(35) Romanucci, V.; Siciliano, A.; Guida, M.; Libralato, G.; Saviano, L.; Luongo, G.; Previtera, L.; Di Fabio, G.; Zarrelli, A. Disinfection by-products and ecotoxic risk associated with hypochlorite treatment of irbesartan. *Sci. Total Environ.* **2020**, *712*, 135625.

641 (36) Schymanski, E. L.; Meinert, C.; Meringer, M.; Brack, W. The use of MS
642 classifiers and structure generation to assist in the identification of unknowns
643 in effect-directed analysis. *Anal. Chim. Acta* **2008**, *615* (2), 136–147.

644 (37) Pikkemaat, M. G.; Dijk, S. O. van; Schouten, J.; Rapallini, M.; van Egmond, H.
645 J. A new microbial screening method for the detection of antimicrobial residues
646 in slaughter animals: The Nouws antibiotic test (NAT-screening). *Food Control*
647 **2008**, *19* (8), 781–789.

648 (38) ISO. ISO 11348-3:1998 - Water quality -- Determination of the inhibitory effect
649 of water samples on the light emission of Vibrio fischeri (Luminescent bacteria
650 test), 1998.

651 (39) Terasaki, M.; Makino, M.; Tatarazako, N. Acute toxicity of parabens and their
652 chlorinated by-products with Daphnia magna and Vibrio fischeri bioassays. *J.*
653 *Appl. Toxicol.* **2009**, *29* (3), 242–247.

654 (40) Leusch, F. D. L.; Neale, P. A.; Busetti, F.; Card, M.; Humpage, A.; Orbell, J.

655 D.; Ridgway, H. F.; Stewart, M. B.; van de Merwe, J. P.; Escher, B. I.
656 Transformation of endocrine disrupting chemicals, pharmaceutical and
657 personal care products during drinking water disinfection. *Sci. Total Environ.*
658 **2019**, *657*, 1480–1490.

659 (41) Jaén-Gil, A.; Castellet-Rovira, F.; Llorca, M.; Villagrasa, M.; Sarrà, M.;
660 Rodríguez-Mozaz, S.; Barceló, D. Fungal treatment of metoprolol and its
661 recalcitrant metabolite metoprolol acid in hospital wastewater:
662 Biotransformation, sorption and ecotoxicological impact. *Water Res.* **2019**,
663 *152*, 171–180.

664 (42) Jaén-Gil, A.; Hom-Diaz, A.; Llorca, M.; Vicent, T.; Blánquez, P.; Barceló, D.;
665 Rodríguez-Mozaz, S. An automated on-line turbulent flow liquid-
666 chromatography technology coupled to a high resolution mass spectrometer
667 LTQ-Orbitrap for suspect screening of antibiotic transformation products
668 during microalgae wastewater treatment. *J. Chromatogr. A* **2018**, *1568*, 57–

669 68.

670 (43) Ferrando-Climent, L.; Gonzalez-Olmos, R.; Anfruns, A.; Aymerich, I.;
671 Corominas, L.; Barceló, D.; Rodriguez-Mozaz, S. Elimination study of the
672 chemotherapy drug tamoxifen by different advanced oxidation processes:
673 Transformation products and toxicity assessment. *Chemosphere* **2017**, *168*,
674 284–292.

675 (44) Sardessai, Y.; Bhosle, S. Tolerance of bacteria to organic solvents. *Res.*
676 *Microbiol.* **2002**, *153* (5), 263–268.

677 (45) Sardessai, Y. N.; Bhosle, S. Industrial potential of organic solvent tolerant
678 bacteria. *Biotechnol. Prog.* **2004**, *20* (3), 655–660.

679 (46) Reemtsma, T.; Fiehn, O.; Jekel, M. A modified method for the analysis of
680 organics in industrial wastewater as directed by their toxicity to Vibrio fischeri.
681 *Fresenius. J. Anal. Chem.* **1999**, *363* (8), 771–776.

682 (47) Dodd, M. C.; Kohler, H. P. E.; Gunten, U. Von. Oxidation of antibacterial

683 compounds by ozone and hydroxyl radical: Elimination of biological activity
684 during aqueous ozonation processes. *Environ. Sci. Technol.* **2009**, *43* (7),
685 2498–2504.

686 (48) Čizmić, M.; Ljubas, D.; Rožman, M.; Ašperger, D.; Ćurković, L.; Babíc, S.
687 Photocatalytic degradation of azithromycin by nanostructured TiO 2 film:
688 Kinetics, degradation products, and toxicity. *Materials (Basel).* **2019**, *16* (6).

689 (49) Radosavljević, K. D.; Lović, J. D.; Mijin, D.; Petrović, S. D.; Jadranin, M. B.;
690 Mladenović, A. R.; Avramov Ivić, M. L. Degradation of azithromycin using
691 Ti/RuO2 anode as catalyst followed by DPV, HPLC-UV and MS analysis.
692 *Chem. Pap.* **2017**, *71* (7), 1217–1224.

693 (50) Paul, T.; Dodd, M. C.; Strathmann, T. J. Photolytic and photocatalytic
694 decomposition of aqueous ciprofloxacin: Transformation products and residual
695 antibacterial activity. *Water Res.* **2010**, *44* (10), 3121–3132.

696 (51) Gomes Júnior, O.; Silva, V. M.; Machado, A. E. H.; Sirtori, C.; Lemos, C. R.;

697 Freitas, A. M.; Trovó, A. G. Correlation between pH and molar iron/ligand ratio
698 during ciprofloxacin degradation by photo-Fenton process: Identification of the
699 main transformation products. *J. Environ. Manage.* **2018**, *213*, 20–26.

700 (52) Zhu, L.; Santiago-Schübel, B.; Xiao, H.; Hollert, H.; Kueppers, S.
701 Electrochemical oxidation of fluoroquinolone antibiotics: Mechanism, residual
702 antibacterial activity and toxicity change. *Water Res.* **2016**, *102*, 52–62.

703 (53) Ou, H. se; Ye, J. shao; Ma, S.; Wei, C. hai; Gao, N. yun; He, J. zhao.
704 Degradation of ciprofloxacin by UV and UV/H2O2 via multiple-wavelength
705 ultraviolet light-emitting diodes: Effectiveness, intermediates and antibacterial
706 activity. *Chem. Eng. J.* **2016**, *289*, 391–401.

707 (54) Yang, B.; Kookana, R. S.; Williams, M.; Ying, G. G.; Du, J.; Doan, H.; Kumar,
708 A. Oxidation of ciprofloxacin and enrofloxacin by ferrate(VI): Products
709 identification, and toxicity evaluation. *J. Hazard. Mater.* **2016**, *320*, 296–303.

710 (55) De Witte, B.; Van Langenhove, H.; Demeestere, K.; Saerens, K.; De

711 Wispelaere, P.; Dewulf, J. Ciprofloxacin ozonation in hospital wastewater
712 treatment plant effluent: Effect of pH and H2O2. *Chemosphere* **2010**, *78* (9),
713 1142–1147.

714 (56) De Bel, E.; Dewulf, J.; Witte, B. De; Van Langenhove, H.; Janssen, C.
715 Influence of pH on the sonolysis of ciprofloxacin: Biodegradability, ecotoxicity
716 and antibiotic activity of its degradation products. *Chemosphere* **2009**, *77* (2),
717 291–295.

718 (57) Yuan, F.; Hu, C.; Hu, X.; Wei, D.; Chen, Y.; Qu, J. Photodegradation and
719 toxicity changes of antibiotics in UV and UV/H2O2 process. *J. Hazard. Mater.*
720 **2011**, *185* (2–3), 1256–1263.

721 (58) Li, M.; Wei, D.; Du, Y. Acute toxicity evaluation for quinolone antibiotics and
722 their chlorination disinfection processes. *J. Environ. Sci.* **2014**, *26* (9), 1837–
723 1842.

724 (59) Tegze, A.; Sági, G.; Kovács, K.; Homlok, R.; Tóth, T.; Mohácsi-Farkas, C.;

725 Wojnárovits, L.; Takács, E. Degradation of fluoroquinolone antibiotics during
726 ionizing radiation treatment and assessment of antibacterial activity, toxicity
727 and biodegradability of the products. *Radiat. Phys. Chem.* **2018**, *147*
728 (February), 101–105.

729 (60) Guo, Q.; Du, Z.; Shao, B. Simulation and experimental study on the
730 mechanism of the chlorination of azithromycin. *J. Hazard. Mater.* **2018**, *359*
731 (July), 31–39.

732 (61) Wang, H.; Hu, C.; Liu, L.; Xing, X. Interaction of ciprofloxacin chlorination
733 products with bacteria in drinking water distribution systems. *J. Hazard. Mater.*
734 **2017**, *339*, 174–181.

735 (62) Dodd, M. C.; Shah, A. D.; Von Gunten, U.; Huang, C. H. Interactions of
736 fluoroquinolone antibacterial agents with aqueous chlorine: Reaction kinetics,
737 mechanisms, and transformation pathways. *Environ. Sci. Technol.* **2005**, *39*
738 (18), 7065–7076.

739 (63) Wang, P.; He, Y. L.; Huang, C. H. Oxidation of fluoroquinolone antibiotics and
740 structurally related amines by chlorine dioxide: Reaction kinetics, product and
741 pathway evaluation. *Water Res.* **2010**, *44* (20), 5989–5998.

742 (64) Domagala, J. M. Structure-activity and structure-side-effect relationships for
743 the quinolone antibacterials. *J. Antimicrob. chemoterapy* **1994**, No. 33, 685–
744 706.

745 (65) Jiao, S.; Zheng, S.; Yin, D.; Wang, L.; Chen, L. Aqueous photolysis of
746 tetracycline and toxicity of photolytic products to luminescent bacteria.
747 *Chemosphere* **2008**, *73* (3), 377–382.

748 (66) Wang, H.; Shi, W.; Ma, D.; Shang, Y.; Wang, Y.; Gao, B. Formation of DBPs
749 during chlorination of antibiotics and control with permanganate / bisulfite
750 pretreatment. *Chem. Eng. J.* **2020**, *392* (October 2019), 123701.

751 (67) Sanchez, J. P.; Domagala, J. M.; Hagen, S. E.; Heifetz, C. L.; Hutt, M. P.;
752 Nichols, J. B.; Trehan, A. K. Quinolone Antibacterial Agents. Synthesis and

753 Structure-Activity Relationships of 8-Substituted Quinoline-3-carboxylic Acids
754 and 1,8-Naphthyridine-3-carboxylic Acids. *J. Med. Chem.* **1988**, *31* (5), 983–
755 991.

756 (68) Higgins, P.; Fluit, A.; Schmitz, F.-J. Fluoroquinolones: Structure and Target
757 Sites. *Curr. Drug Targets* **2005**, *4* (2), 181–190.

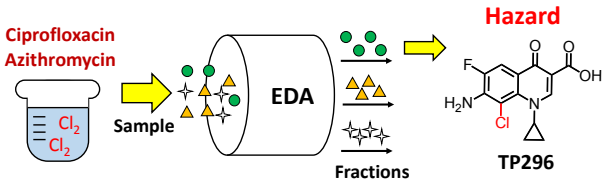
758 (69) Escher, B. I.; Neale, P. A.; Villeneuve, D. L. The advantages of linear
759 concentration–response curves for in vitro bioassays with environmental
760 samples. *Environ. Toxicol. Chem.* **2018**, *37* (9), 2273–2280.

761 (70) Müller, M. E.; Vikstrom, S.; König, M.; Schlichting, R.; Zarfl, C.; Zwiener, C.;
762 Escher, B. I. Mitochondrial Toxicity of Selected Micropollutants, Their
763 Mixtures, and Surface Water Samples Measured by the Oxygen Consumption
764 Rate in Cells. *Environ. Toxicol. Chem.* **2019**, *38* (5), 1000–1011.

765

766

Graphical Abstract



778

779

780

781

782

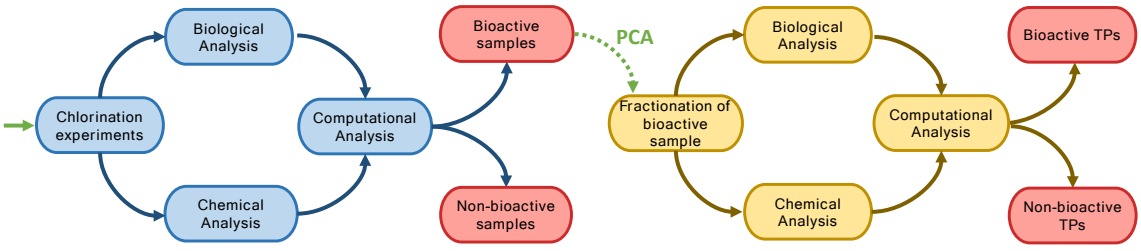
783

784

785

786

787



788

789 **Figure 1.** Adapted effect-directed analysis workflow used in this study.

790

791

792

793

794

795

796

797

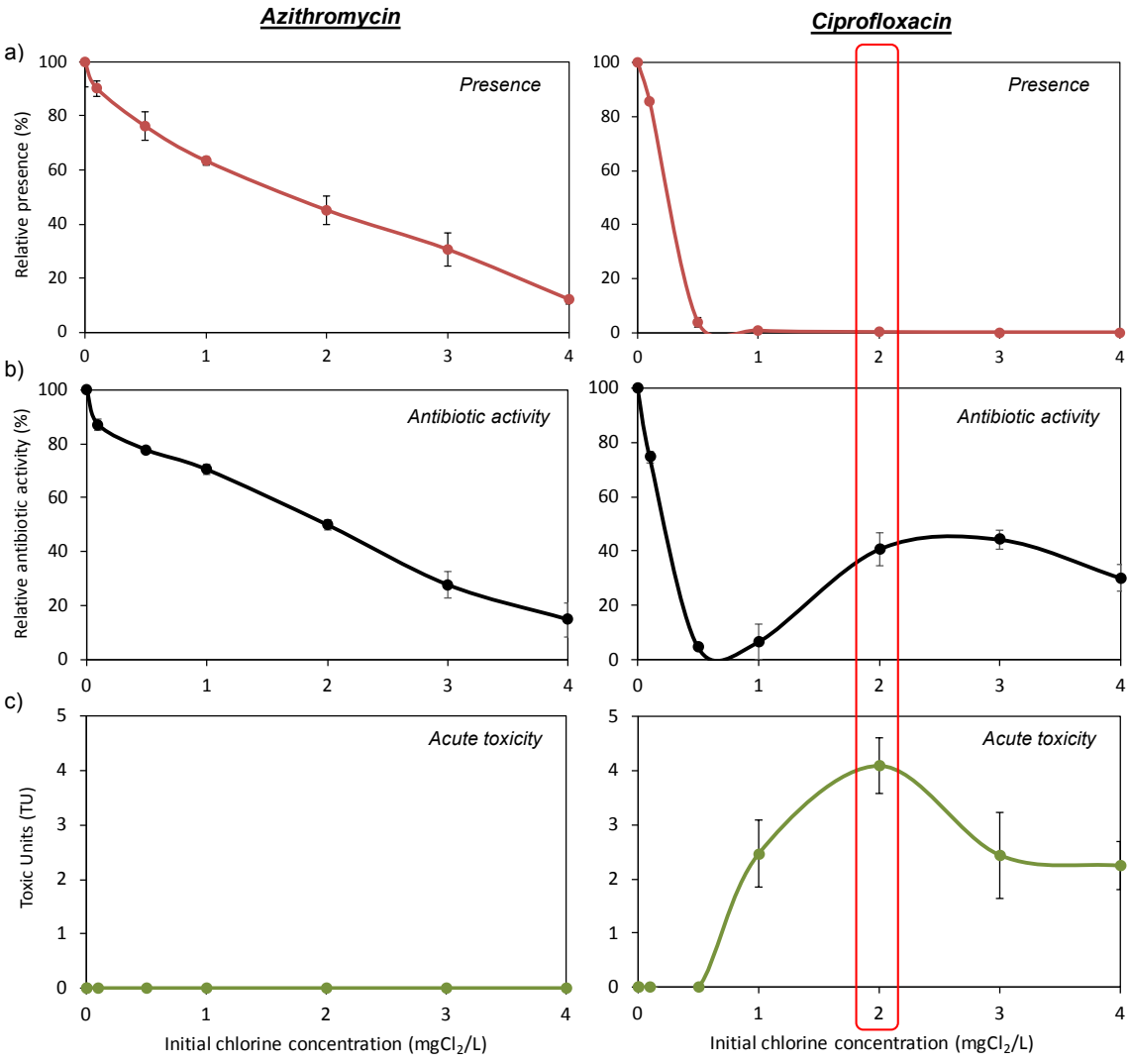
798

799

800

801

802



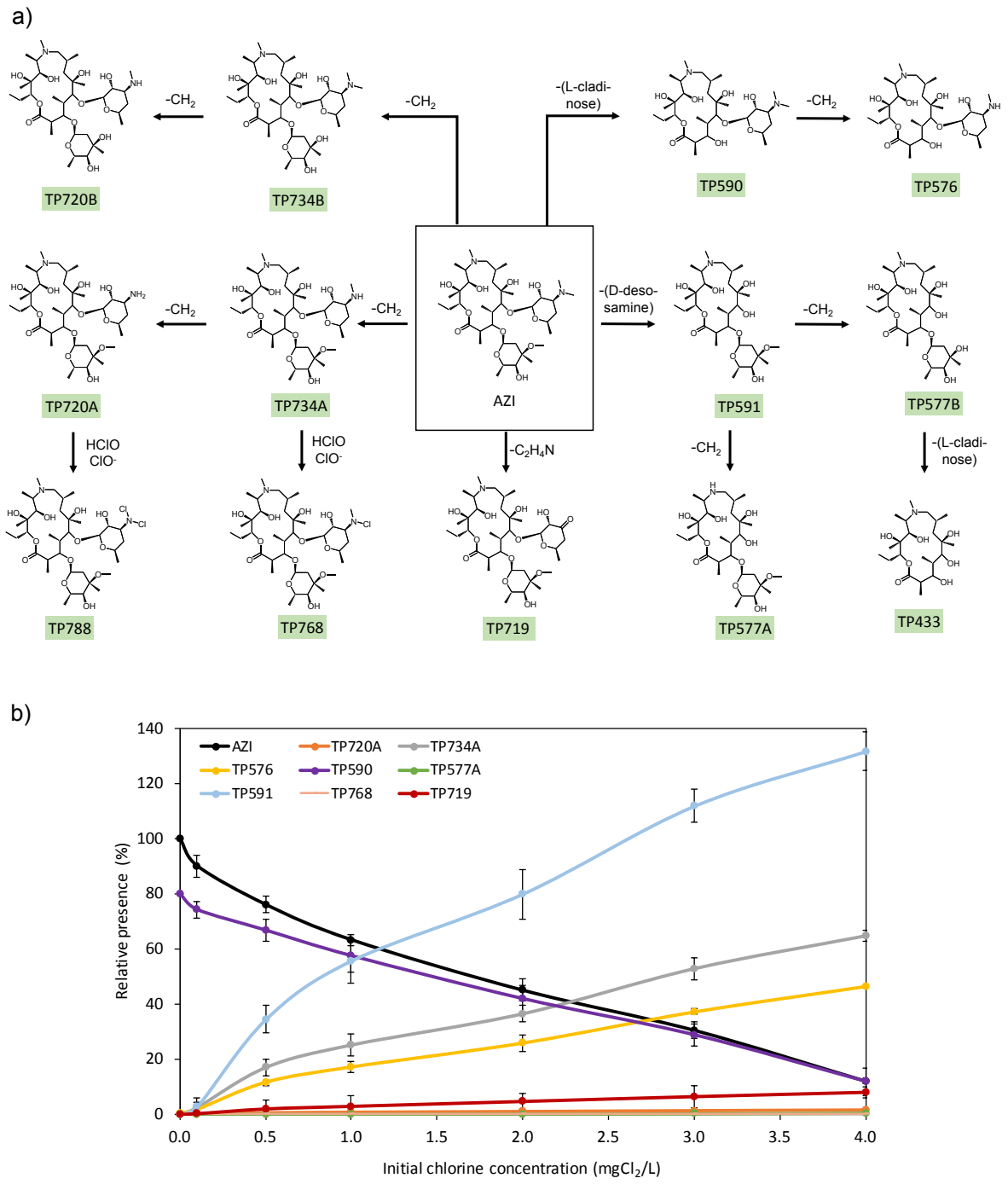
803

804 **Figure 2.** Monitoring of (a) the degradation of spiked antibiotics (azithromycin or

805 ciprofloxacin), (b) antibiotic activity and (c) acute toxicity after 24h of treatment in

806 chlorination experiments at different initial chlorine dose. Relative values of presence
807 and antibiotic activity were calculated with respect to the values measured at the
808 initial time. In the case of acute toxicity is calculated in toxic units (TU). The red
809 square indicates the sample selected for further sample fractionation.

810



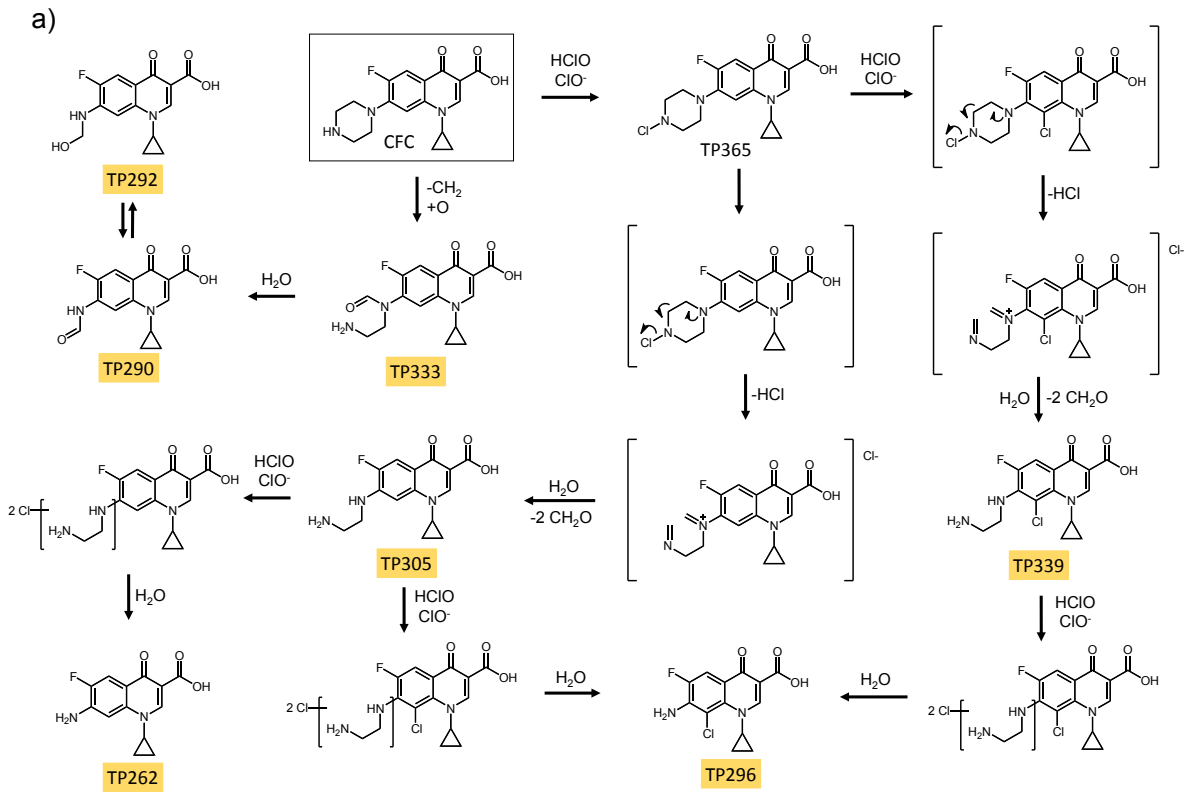
811

812 **Figure 3.** a) Suggested transformation mechanism of azithromycin in chlorination

813 experiments. In green, tentatively identified intermediates detected in this study. b)

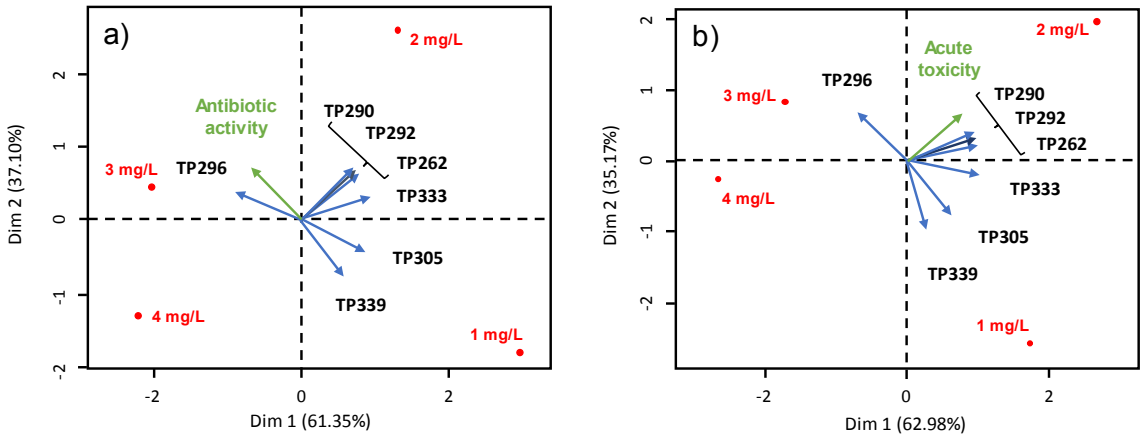
814 The relative presence of the most representative intermediates identified after

815 chlorination experiments at different chlorine dose.



816

817 **Figure 4.** a) Suggested transformation mechanism of ciprofloxacin in chlorination
818 experiments. In yellow, tentatively identified intermediates detected in this study. b)
819 The relative presence of the intermediates identified after chlorination experiments
820 at different chlorine dose. The red square indicates the sample selected for further
821 sample fractionation.



822

823 **Figure 5.** PCA loadings of the presence of the intermediates identified regarding the
824 a) antibiotic activity and b) acute toxicity tested in chlorination samples.

825

826

827

828

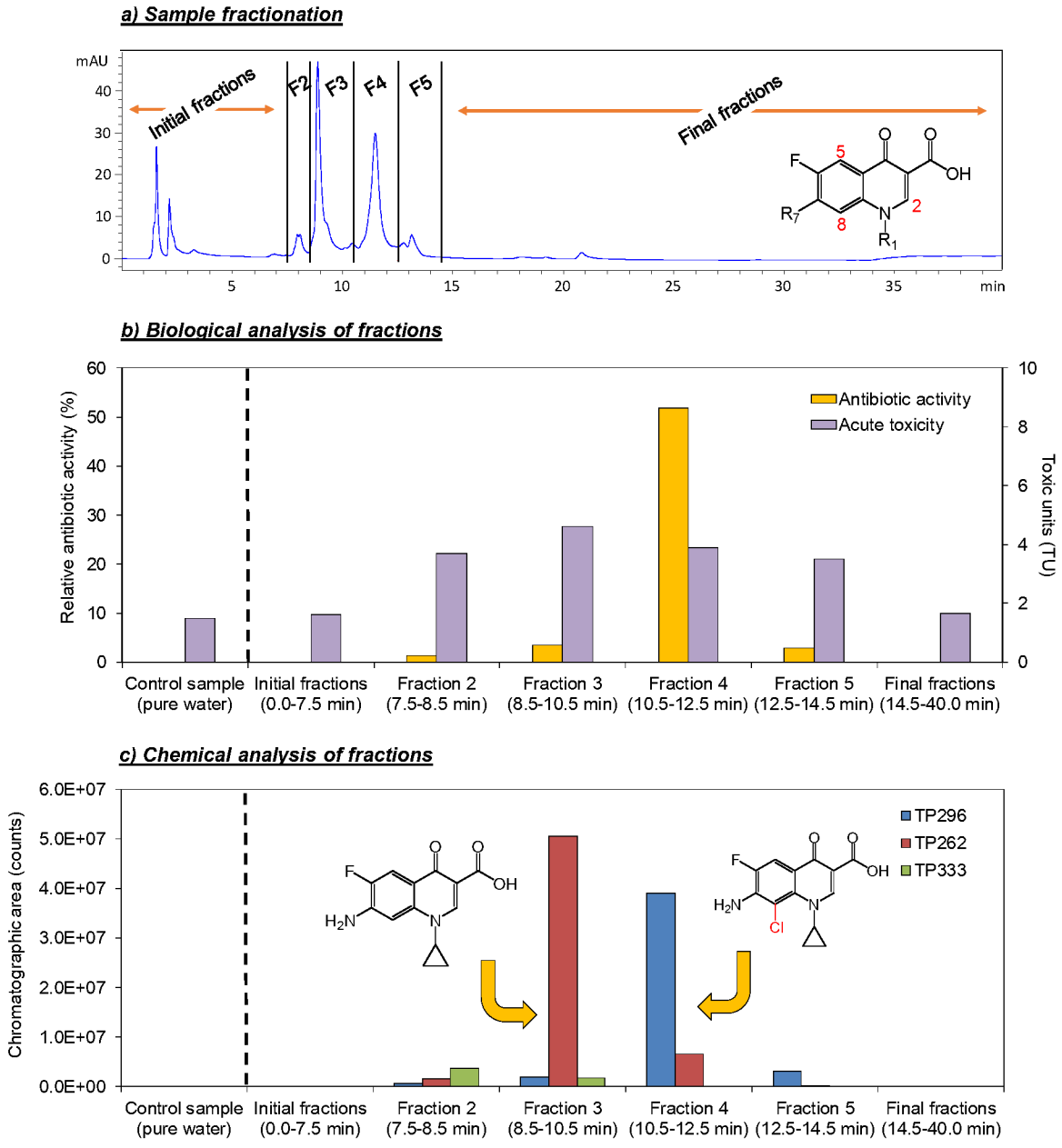
829

830

831

832

833



834

835 **Figure 6.** a) Fractionation of the selected bioactive sample (10 mgCl_2/L vs 10 mg/L

836 CFC at the initial time); b) acute toxicity (calculated as EC_{10} and expressed in TU)

837 and relative antibiotic activity of fractions collected and control samples; and c)

838 chemical analysis (chromatographic area) of fractions and control samples.