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Occurrence and behavior of macrolide antibiotics in municipal wastewater treatment: Possible importance of metabolites, synthesis by-products and transformation products

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2 Possible importance of metabolites, synthesis by-products and transformation products

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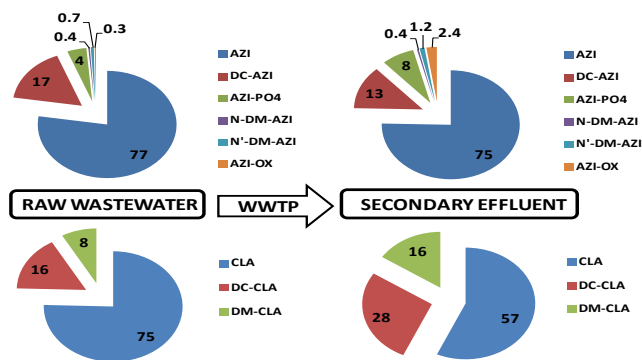
17 **Abstract**

18 A one-year study on the occurrence and fate of macrolide antibiotics and their metabolites, synthesis
19 by-products and transformation products (TPs) was performed in the wastewater treatment plant of
20 the city of Zagreb (Croatia). The target compounds were found in all analyzed influent and effluent
21 samples, with the total concentrations of azithromycin-, clarithromycin- and erythromycin-related
22 compounds reaching up to 25, 12 and 0.25 $\mu\text{g/L}$, respectively. The most prominent individual
23 constituents were the parent macrolides azithromycin and clarithromycin. However, a substantial
24 contribution of their derivatives, formed by deglycolysation and microbial phosphorylation, was also
25 detected. In addition, widespread presence of several linearized non-target TPs was confirmed for
26 the first time in real wastewater samples by suspect screening analysis. Complex characterization of
27 macrolide-derived compounds enabled decoupling of industrial and therapeutic sources from the *in-*
28 *situ* transformations. Due to the high inputs, incomplete removal and/or formation of several TPs
29 during the conventional wastewater treatment, the average mass load of azithromycin-related
30 compounds in secondary effluents exceeded 3.0 g/day/1000 inhabitants. This is the first study to
31 reveal the importance of metabolites, by-products and TPs for the overall mass balance of macrolide
32 antibiotics in urban wastewater systems.

33

34 **Keywords:** azithromycin, erythromycin, clarithromycin, intermediates, metabolites, transformation
35 products

36 Abstract art



37

38

40 1. Introduction

41 Antibiotics are widespread in the environment and represent one of the most extensively studied
42 classes of emerging organic contaminants of pharmaceutical origin. Continuous exposure to
43 antibiotics in the environment can cause different negative effects on non-target species,¹ however a
44 special concern has been raised regarding possible contribution of antibiotic residues to the
45 development and proliferation of the resistant bacteria, which is one of the major global threats to
46 human health in the near future.² The ubiquitous occurrence of antibiotics in different environmental
47 compartments has been confirmed in numerous studies in the last 20 years.³⁻⁶ Most of these studies
48 pointed out urban wastewater as the major global source of antibiotics in the environment. Due to
49 the incomplete removal of many classes of antibiotics during conventional and/or advanced
50 wastewater treatment,⁷⁻¹¹ significant quantities of antibiotic residues are continuously released into
51 the receiving ambient waters and soil environments. However, most of the studies published so far
52 were focused only on parent antibiotics,¹² or, at best, included some major metabolites.^{4,5,13} On the
53 other hand, the literature on the occurrence of transformation products (TPs) and synthesis by-
54 products in the aquatic environment is very scarce¹⁴⁻¹⁷ and their contribution to the overall mass
55 balance of antibiotic-derived compounds remains largely unknown.

56 Among several classes of recalcitrant antibiotics, macrolides recently came under special scrutiny.
57 Their main representatives - erythromycin (ERY), clarithromycin (CLA) and azithromycin (AZI) - have
58 been included in the EU Watch List of potentially hazardous compounds for the aquatic
59 environment.¹⁸ Widespread occurrence of macrolide antibiotics in urban wastewater, as well as their
60 incomplete removal during wastewater treatment, has been frequently reported.^{5,19,20} Negative
61 removal rates, observed in some of the studies,^{13,21} indicated possible significance of metabolites
62 and/or TPs to the overall mass loads of macrolides in urban wastewater. Moreover, it was shown
63 that the transformation processes in the environment, including both biotic²²⁻²⁵ and abiotic
64 degradation²⁶⁻²⁹ can lead to formation of number of TPs, whose ecotoxicological properties are

65 largely unknown. Several TPs of macrolide antibiotics, including those formed by opening macrolide
66 ring, were recently found to be generated during microalgae wastewater treatment.²⁵ In our earlier
67 study, novel TPs of AZI and roxythromycin (ROX), formed by microbial phosphorylation, were
68 identified in wastewater effluent of a membrane bioreactor,³⁰ while a recent study on aerobic
69 biodegradation of AZI, CLA and ERY revealed a number of previously unreported, novel TPs and
70 allowed proposing a general transformation pathways scheme for AZI.³¹ In addition, non-target
71 analysis of freshwater sediments polluted by pharmaceutical industry effluent indicated the
72 importance of intermediates in AZI synthesis.³² In order to address the complexity of macrolide-
73 derived compounds, we recently developed a novel quantitative analytical method, comprising
74 parent macrolide antibiotics, their major human metabolites and TPs, as well as by-products from AZI
75 synthesis, in wastewater and river water samples.¹⁷ This preliminary study clearly demonstrated the
76 possible importance of non-parent macrolide compounds in municipal wastewater and industrial
77 effluents. The concentrations of several metabolites and/or TPs of AZI and CLA were detected in
78 municipal wastewater in similar concentration ranges as the parent antibiotics, while wastewater
79 effluents from pharmaceutical industry were characterized by elevated concentrations of by-
80 products from AZI synthesis.¹⁷

81 The aim of this work was to perform a detailed study on the occurrence and fate of macrolide-
82 related compounds in the central wastewater treatment plant (WWTP) of the city of Zagreb, covering
83 a period of one year and encompassing both raw wastewater (RW) and biologically treated
84 wastewater (secondary effluent; SE). The relevance of the applied comprehensive approach, which
85 for the first time included an extended range of AZI-, ERY- and CLA-related compounds, was not only
86 to demonstrate the need to include non-parent compounds in the overall risk assessment, but also to
87 provide the clues for decoupling different sources based on the composition of macrolide
88 constituents in a typical urban wastewater facility receiving mixed domestic and industrial
89 wastewater.

90

91 **2. Experimental**

92 **2.1. Chemicals and materials**

93 The target analytes included: AZI, decladinosyl azithromycin (DC-AZI), phosphorylated azithromycin
94 (AZI-PO₄), *N*-demethyl azithromycin (N-DM-AZI), *N'*-demethyl azithromycin (N'-DM-AZI),
95 azithromycin *N*-oxide (AZI-OX), anhydro erythromycin (ERY-H₂O), erythromycin enol ether (ERY-EE),
96 *N*-demethyl erythromycin (DM-ERY), erythromycin imino ether (ERY-IE), erythromycin oxime (ERY-
97 OX), CLA, *N*-demethyl clarithromycin (DM-CLA), decladinosyl clarithromycin (DC-CLA) and ROX.
98 Deuterated azithromycin (AZI-*d*₃) and clarithromycin (CLA-*d*₃) were used as internal standards. The
99 structures and abbreviations of the target analytes included in the quantitative analytical method are
100 presented in Figure S1 in Supporting Information (SI). Their reference standards were purchased
101 from Toronto Research Chemicals (Canada), with the exception of AZI, ERY-IE, ERY-OX and CLA, which
102 were kindly supplied by Pliva (Zagreb, Croatia), ROX, which was acquired from Sigma-Aldrich
103 (Germany), and AZI-PO₄, which was prepared in our laboratory by microbial transformation of AZI
104 (details can be found in Senta et al., 2017).¹⁷

105 HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from BDH Prolabo (UK) and
106 ACS grade formic acid from Sigma-Aldrich. Elix-Milli-Q system (Millipore, USA) was used for the
107 production of ultrapure water. Solid-phase extraction (SPE) cartridges Oasis HLB (200 mg/6 mL) and
108 Strata SAX (100 mg/3 mL), were obtained from Waters (USA) and Phenomenex (USA), respectively,
109 whereas glass-fiber filters (GF/D) were delivered by Whatman (UK). HPLC column ACE C18 PFP (150 ×
110 3 mm; 3 μm) was purchased from Advanced Chromatography Technologies (UK), whereas UPLC
111 column Acquity BEH C₁₈ (50 mm × 2.1 mm; 1.7 μm) was purchased from Waters.

112

113 **2.2. Selection of the target compounds**

114 Our study was focused on three most prominent macrolide antibiotics - AZI, ERY and CLA, which were
115 recently included in the EU Watch list of potential surface water pollutants.¹⁸ Moreover, these
116 substances account for almost entire consumption of macrolide antibiotics in Croatia. The macrolide
117 consumption in 2014, expressed in defined daily doses (DDD) per 1000 inhabitants was 1.6, 0.9 and
118 <0.2 for AZI, CLA and ERY, respectively.³³ In addition, several other macrolide compounds related to
119 AZI, ERY and CLA (Figure S1) were also included in quantitative analyses. These additional macrolide-
120 derived compounds can be classified into three categories as production intermediates and/or by-
121 products, human metabolites, and transformation products (TPs), predominately formed in
122 wastewater itself.

123 It should be noted that parent ERY is very unstable at $\text{pH} \leq 7$ and converts rapidly to ERY-H₂O, its
124 main metabolite and TP.³⁴ This conversion is especially efficient at low pH, such as in stomach of the
125 patients treated with this antibiotic.³⁵ This conversion also occurs in the environment, as well as
126 during the sample preparation and analysis and, therefore, most of the methods for determination of
127 ERY in environmental samples include ERY-H₂O rather than parent ERY.^{13,19-21,34} In addition to ERY-
128 H₂O, the present study included ERY-EE, which was shown to be TP formed during biodegradation,²²
129 as well as DM-ERY, which can be either metabolite formed by demethylation in the liver³⁶ or
130 microbial TP.³¹

131 Special attention was given to AZI, a semi-synthetic macrolide antibiotic produced from ERY. AZI was
132 discovered in Croatia almost 40 years ago and has been manufactured in large quantities in the
133 facilities located near the city of Zagreb for more than 30 years. Our study included all three
134 intermediates in AZI synthesis - ERY-OX, ERY-IE and N-DM-AZI, as well as a suite of human
135 metabolites and/or TPs (DC-AZI, N'-DM-AZI, AZI-PO₄ and AZI-OX). DC-AZI and N'-DM-AZI are human
136 metabolites of AZI, but they can also be formed as impurities during AZI synthesis and by *in-situ*
137 transformations in wastewater. On the other hand, AZI-PO₄ and AZI-OX are classified as TPs, although
138 traces of AZI-OX can be formed during AZI synthesis as well.

139 Two CLA-related compounds were included in the quantitative analytical method. DM-CLA is either
140 human metabolite of CLA³⁷ or microbial TP,³¹ while DC-CLA, similar as DC-AZI, can be either by-
141 product of CLA synthesis or minor TP formed in wastewater itself. Unfortunately, due to the lack of a
142 reference standard, major CLA metabolite, 14-hydroxy clarithromycin (CLA-OH),³⁷ could be
143 determined only semi-quantitatively.

144 Finally, another macrolide antibiotic, ROX, which is not included in the EU Watch list, was also
145 analyzed, but only as a parent compound.

146

147 **2.3. Sampling**

148 24-h composite wastewater samples, including RW and SE, were collected in the central WWTP of
149 the city of Zagreb (Croatia). All samples were time-proportional and were collected from 8 a.m. of
150 the previous day to 8 a.m. of the sample collection day, with the sampling time interval of 15 min.
151 WWTP of the city of Zagreb is a full-scale mechanical-biological treatment plant which receives a
152 combined municipal and industrial wastewater, including pharmaceutical industry effluents. It has
153 designed capacity of 1.2 million population equivalents, but currently serves approximately 700,000
154 inhabitants. The average hydraulic load of raw wastewater is about 300,000 m³/day and hydraulic
155 retention time (HRT) is approximately 12 h. Additional details about the WWTP can be found in SI
156 (Table S1).

157 Collection of wastewater samples was performed using two sampling schemes. The main set of
158 samples was collected over a period of approximately one-year (February to December 2017), by
159 carrying out regular sampling on Monday and Wednesday of the same week, with a frequency once a
160 month. The second scheme included collection of the wastewater samples during 7 consecutive days
161 (Wednesday – Tuesday), covering a period of 1 week (from 22nd to 28th March 2017). In total, 29 RW
162 and 29 SE samples were collected.

163

164 **2.4. Sample treatment**

165 All wastewater samples collected within the one-year sampling interval were processed shortly after
166 being brought back to the laboratory (typically 1–2 hours from sampling). Samples collected within
167 the one-week sampling interval were frozen immediately after collection and processed later. All
168 samples were treated according to the previously developed analytical procedure.¹⁷ A brief
169 description of the applied analytical procedure is provided in SI.

170 In addition to wastewater samples, pills containing 500 mg of either AZI or CLA were also analyzed.
171 Each pill was first weighted and grained. A small aliquot was then dissolved in MeOH to prepare stock
172 solutions containing 1 mg/mL of active ingredient (AZI or CLA). Stock solutions were then diluted with
173 100 mM ammonium formate/MeOH (1/1, v/v) for instrumental analysis.

174

175 **2.5. Instrumental analyses**176 **2.5.1. Quantitative LC-MS/MS analysis**

177 Our previously developed analytical method,¹⁷ based on liquid chromatography coupled to tandem
178 mass spectrometry (LC-MS/MS), was used for quantitative instrumental analysis. Target compounds
179 were separated on ACE C₁₈ PFP HPLC column, using gradient elution with 0.1% formic acid in water
180 (v/v) and ACN as eluents. Detection and quantification were performed on a TSQ Quantum triple
181 quadrupole instrument (Thermo Electron, USA), using multiple reaction monitoring (MRM) mode in
182 positive polarity, with the two characteristic transitions selected for each target compound. First
183 transition was used for quantification, whereas second transition, together with the ratio of the two
184 transitions, was used for confirmation. The developed procedure allowed reliable determination of
185 all target compounds included in the quantitative analytical method, with high accuracy ($\geq 75\%$) and

186 repeatability ($RSD \leq 5\%$) and low method quantification limits (MQL) (2–32 ng/L). All operational
187 parameters, as well as detailed performance of the analytical method, can be found elsewhere.¹⁷ The
188 method validation parameters for matrices included in this study are also given in SI (Table S2).

189

190 *2.5.2. Qualitative and semi-quantitative LC-MS analyses*

191 Additional, qualitative analyses of wastewater samples were performed by suspect screening of the
192 extracts for a presence of the number of additional AZI and CLA TPs previously identified in model
193 biotransformation experiments performed with the enriched sludge culture.³¹ Their structures can be
194 found in SI (Figure S2 and S3). The screening was performed using ultrahigh-performance liquid
195 chromatography (UHPLC) coupled to quadrupole-time-of-flight mass spectrometry (QToF MS), by
196 applying the chromatographic and mass-spectrometric conditions given in Terzic et al. (2018).³¹ The
197 identification of the additional TPs was made based on the accurate mass of protonated molecules
198 and the retention time using samples from the model biotransformation experiments³¹ as qualitative
199 reference standards. Furthermore, additional confirmation and approximative semi-quantitative
200 determination of these additional TPs was performed by triple quadrupole LC-MS/MS system. Due to
201 the lack of reference standards, their MRM transitions (Table S3) were selected manually, based on
202 MS2 spectra recorded in above mentioned model biotransformation experiments,³¹ whereas the
203 applied chromatographic conditions were identical to those applied for the quantitative MRM
204 determinations of commercially available analytes.¹⁷

205

206 **3. Results and discussion**

207 ***3.1. Occurrence of macrolide-derived compounds in raw wastewater and secondary effluent***

208 An overview of the occurrence of the target macrolide compounds in wastewater samples is
209 presented in Table 1. Most of the analytes were found in all analyzed RW samples. The most
210 prominent parent macrolide antibiotic was AZI, with concentrations ranging from 0.27 to 22.7 $\mu\text{g/L}$
211 (average $5.8 \pm 5.3 \mu\text{g/L}$), which is generally an order of magnitude higher than the typical
212 concentration ranges determined in municipal wastewater in similar studies^{4,9,38,39} Other AZI-related
213 substances were also detected in all or in the majority of RW samples. The most abundant ones were
214 DC-AZI (0.087–7.5 $\mu\text{g/L}$) and AZI- PO_4 (0.049–1.2 $\mu\text{g/L}$), whereas concentrations of other AZI-related
215 compounds, especially specific markers of AZI-synthesis, were much lower ($< 100 \text{ ng/L}$). Such
216 distribution of AZI-related compounds was rather different from that found in the wastewater
217 effluent receiving wastewater inputs from AZI-synthesis facility.¹⁷ The concentrations of CLA in
218 wastewater of the city of Zagreb were significantly lower than the concentrations of AZI. In most of
219 the RW samples CLA levels were below 1 $\mu\text{g/L}$ (range 111–571 ng/L), with exception of two samples
220 collected in April, when elevated concentrations (10 and 3.6 $\mu\text{g/L}$) were determined. These
221 concentration ranges are in line with most of the previous studies.^{4,6,9,38,39} Both CLA-related
222 compounds included in quantitative target analyses (DC-CLA and DM-CLA) were also determined in
223 all analyzed samples, but their concentrations were consistently lower than the concentrations of
224 parent CLA. For ERY-related compounds the concentrations were even lower, indicating significantly
225 lower consumption of ERY in human therapy. The concentrations of ERY- H_2O and EEE, as the
226 prevalent ERY-related compounds, were typically below 50 ng/L and 20 ng/L , respectively, with only
227 few exceptions observed in March. Moreover, only traces of possible ERY TPs, such as DM-ERY, were
228 occasionally detected, but the concentrations were always below the MQL. The concentrations of
229 ERY- H_2O were either similar or lower than the concentrations determined in other studies.^{9,19,20}
230 Finally, in our survey, ROX was not detected in any of the analyzed samples, which is in accordance
231 with its very limited usage in Croatia,³³ as well as with the previous data.⁵ In qualitative terms, our
232 results are in accordance with the use of macrolide antibiotics in Croatia (AZI>CLA>ERY>ROX).

233 It should be stressed that all compounds determined in RW were also found in SE, with similar
234 detection frequencies and concentration ranges. In fact, concentrations of some substances were
235 often higher in SE than in RW samples, which is further discussed in Section 3.3.

236 Figure 1 shows the average relative contribution of each individual compound to the total loads of
237 AZI- and CLA-related substances in RW and SE and clearly illustrates the importance of non-parent
238 compounds to the mass balance of the target macrolides. Already in RW, the contribution of
239 metabolites, by-products and TPs reached a significant percentage of approximately 25% for both
240 macrolides. It is interesting to note that after secondary treatment the contribution of non-parent
241 macrolides remained almost the same for AZI but significantly increased (44%) for CLA. Along these
242 lines, it should be stressed that the major human metabolite, CLA-OH, was not included in this
243 calculation, due to the lack of a quantitative reference standard, however our semi-quantitative
244 estimates indicate that inclusion of CLA-OH would further shift the mass balance towards the
245 predominance of non-parent CLA compounds. This is particularly important when we consider that
246 CLA-OH retains the antibiotic activity after the transformation from CLA.³⁷

247

248 **3.2. Mass loads and decoupling of possible sources of macrolides in RW**

249 Mass loads of the target compounds were calculated by multiplying their concentrations by the
250 volume of wastewater passing daily through the WWTP. Mass loads of AZI- and CLA-related
251 compounds in RW are presented in Figure 2 (one-year sampling interval) and Figure 3 (one-week
252 sampling interval). It should be noted that in these Figures parent antibiotics are presented
253 individually, whereas the mass loads of related substances (metabolites, by-products and TPs) are
254 summed up. The individual mass loads of all major AZI- and CLA-related compounds determined
255 during the one-year sampling interval are additionally presented in Figure S4 and S5 (SI), respectively.

256 Figure 2 shows that the mass loads of parent AZI were markedly variable (88 to 6861 g/day), with
257 several distinctive peaks throughout the year. The average value of 1861 ± 1744 g/day is an order of
258 magnitude higher than the typical mass loads (121 ± 64 g/day) determined in the investigated WWTP
259 in our previous study.⁵ A rather similar one-year temporal pattern was also observed for DC-AZI, AZI-
260 PO₄ and AZI-OX (Figure S4, SI), but their mass loads were comparatively lower (323 ± 485 , 111 ± 77
261 and 8.2 ± 9.7 g/day, respectively).

262 Substantial increase in the mass loads of AZI (15 times), compared to our previous study performed
263 in 2009,⁵ cannot be linked to the increased use of this antibiotic in human medicine. According to the
264 official statistics, consumption of AZI in the last decade increased only about 30%³³ and can be
265 estimated at approximately 350 g/day for the city of Zagreb, which is 5 times lower than the average
266 mass load determined in our study, even if we ignore metabolic transformation of AZI, which would
267 make this difference even more pronounced. Moreover, if human consumption is supposed to be the
268 main source of input of this antibiotic in wastewater, the observed extreme variability of AZI mass
269 loads (over two orders of magnitude during a single year) would be highly unlikely. Furthermore, it is
270 even more difficult to associate the variability of the mass loads of over one order of magnitude
271 during a single week (Figure 3) with the therapeutic use of AZI. For example, our previous studies on
272 opioid analgesics in the same WWTP indicated a rather small variability of therapeutic consumption
273 during the week.⁴⁰ These findings suggest a direct disposal as the main source of input of AZI in the
274 investigated WWTP during the peak concentrations. Having in mind that investigated WWTP receives
275 mixed domestic and industrial wastewaters, possible source of the enhanced AZI mass loads might
276 have been local pharmaceutical industry, which produces substantial amount of macrolide
277 antibiotics, especially AZI. Since the facility for AZI synthesis is located outside of the city of Zagreb
278 and does not discharge its wastewater to the sewer system of the city, this indicated that some other
279 pharmaceutical industry processes, such as formulation and packing of macrolide active ingredients,
280 represented the most likely additional source of AZI in the investigated WWTP. Moreover, it was
281 shown that the industrial effluents from the AZI synthesis facility contain highly elevated

282 concentrations of AZI synthesis intermediates (up to 5.7 mg/L),¹⁷ whereas the concentrations of
283 these compounds in the municipal wastewater samples of the city of Zagreb, analyzed in this study
284 were rather low (N-DM AZI: <MQL–70 ng/L; ERY-OX: <MQL–12 ng/L; ERY-IE: <MQL). Disposal of
285 unused medications directly into the sewer system as a possible alternative source of occasionally
286 high loads of AZI does not seem very likely.

287 The mass loads of CLA in most of the RW samples were between 28 and 188 g/day, which is generally
288 comparable with the previously reported data for this WWTP.⁵ However, much higher mass loads,
289 2798 and 1019 g/day, were determined in two samples collected in April. A very similar temporal
290 pattern, with distinctive peak in April (416 and 284 g/day), was also observed for DC-CLA (Figure S5,
291 SI). This highly irregular feature indicated the existence of an additional source of CLA during that
292 short period. Otherwise, the mass loads of CLA were much less variable than the mass loads of AZI,
293 both within the investigated one-year (Figure 2) and one-week sampling interval (Figure 3) and were
294 in line with the mass loads which can be estimated from human therapeutic use.³³ The exceptional
295 stability of CLA levels in one-week survey (RSD 7%) clearly indicated that the variability of human
296 consumption within a week was less than 10% and, therefore, sudden changes must have been a
297 consequence of an additional non-therapeutic input. Thus, the observed extreme increase of CLA
298 mass loads (Figure 2), which was evidenced in April, was interpreted as an occasional release of CLA
299 from the pharmaceutical facilities.

300 Mass loads of ERY-H₂O (2.4–37 g/day) were similar as in our previous study⁵ and most probably
301 reflected a rather restricted human consumption of ERY in Croatia,³³ although possible contribution
302 from veterinary medicine cannot be excluded. The contribution of other ERY-related compounds to
303 the total mass loads was even lower.

304 In order to estimate the possible contribution of non-parent macrolide compounds from formulation
305 facilities, we analyzed AZI and CLA pills from the local manufacturer for the main impurities. Their
306 mass fractions were calculated as percentages of the mass of the corresponding parent antibiotic in

307 the pill. The results, presented in Table S4 (SI), show that several AZI- and CLA-related compounds
308 determined in RW were also detected in the analyzed pills. In the RW samples characterized by
309 largely enhanced concentrations of AZI and CLA, the contribution of impurities, such as N-DMA, N'-
310 DMA and AZI-OX, was typically lower than 1%, which was rather close to the levels in the analyzed
311 pills. However, the average percentages of N-DMA, N'-DMA and AOX in all other RW samples were
312 1.2%, 1.6% and 0.5%, respectively, indicating a significant contribution of therapeutic consumption
313 and/or formation in the sewer system after disposal. It should be stressed that the average
314 percentages of DC-AZI and DC-CLA were rather high (17% and 16%, respectively), exceeding the
315 percentages found in pills more than 100 times. Therefore, there is a strong indication that these
316 decladinosyl derivatives originated primarily from an additional formation in the sewer. We assume
317 that this hypoxic environment could harbor bacteria which contain enzymes capable of transforming
318 macrolide antibiotics, as recently suggested in the study by Gonzalez-Gil et al.⁴¹ Along these lines, it
319 should be stressed that characteristic phosphorylated TPs were not detected in the pills, while their
320 contribution in RW reached an average of 8.8%. Therefore, it seems that phosphorylated macrolides
321 can be used as exclusive indicators of their microbial transformations in the wastewater itself. On the
322 other hand, most of the remaining substances can have multiple sources, which makes their
323 exclusive assignment to individual classes (metabolites, TPs and by-products) very difficult.

324 Seasonal trends of the mass loads of AZI and CLA-related compounds were also investigated, and the
325 results are presented in Fig S6 A (SI). Although some differences were observed, they were not
326 statistically significant, due to the high variability of the mass loads in the investigated period, caused
327 by non-therapeutic sources, especially for AZI. However, if the mass loads of CLA determined in April
328 were excluded, a clear seasonal trend, with the significantly lower loads (one-way ANOVA; $p < 0.001$)
329 during the summer was obtained for this compound (Fig S6 B, SI), which is in line with the previous
330 studies.⁴²⁻⁴⁴ This can be explained with the lower consumption of macrolide antibiotics, which are
331 mostly used to treat respiratory infections, during summer months. This observation additionally
332 supports the hypothesis that the main source of CLA in the investigated WWTP is human

333 consumption, with exception of the two samples collected in April, when direct disposal from
334 pharmaceutical industry greatly surpassed the contribution from human consumption.

335

336 ***3.3. Behavior of macrolide-derived compounds during activated sludge treatment***

337 As already pointed out in Section 3.1., the concentrations of several macrolide substances were
338 similar or even higher in SE than in RW, indicating their incomplete removal during conventional
339 wastewater treatment. However, it should be noted that HRT in the investigated WWTP is rather
340 short (12 h), which may hamper the accurate determination of the removal efficiency when 24-hours
341 samples are collected, especially in the case of frequent pulse discharges from pharmaceutical
342 facilities, causing high diurnal variability of the target compounds in wastewater. Therefore, removal
343 rates calculated from 24-hour composite samples collected on the same date were found to be
344 inaccurate and highly variable. Nevertheless, although removal rates of the individual target
345 compounds could not be accurately determined for the samples collected in the one-year sampling
346 interval, figures showing their mass loads in RW and SE (Figure S4 and S5, SI) can provide valuable
347 additional information on their behavior during conventional wastewater treatment at a larger time-
348 scale, covering different seasons.

349 To obtain more robust and reliable results, the removal efficiencies of the target macrolides were
350 assessed based on the weekly mass balance exercise carried out during seven consecutive days. The
351 results on the weekly mass loads in RW and SE and the removal efficiencies for the major macrolide-
352 derived compounds are presented in Table 2. This approach successfully compensated the
353 inconsistencies associated with the industrial pulse of AZI from 24th March. Table 2 shows that CLA
354 was the only major macrolide with elimination above 50%. Removal efficiency for this substance was
355 very similar as in our previous study in the same WWTP⁵ and in line with the findings in the one-year
356 sampling interval. Partial removal was also obtained for DM-CLA, as well as for ERY-H₂O, which
357 removal rate was slightly higher than in the previous study, but still below 40%. On the other hand,

358 virtually no removal was observed for AZI, AZI-PO₄ and DC-CLA. Low removal of parent AZI in the
359 investigated WWTP has been already reported in the literature.⁵ On the other hand, negative
360 removal rates were obtained for DC-AZI and N'-DM-AZI, whose mass loads were approximately 2.5
361 times higher in SE than in RW during the one-week sampling interval. This was even more
362 pronounced for AZI-OX, which showed an order of magnitude higher mass loads in SE than in RW.
363 Rather similar features for AZI-OX and N'-DM-AZI were also observed during the one-year sampling
364 interval (Figure S4, SI). This suggested possible formation of these TPs during conventional
365 wastewater treatment. On the other hand, results for DC-AZI were less consistent. In fact, in some
366 cases, mostly during the pulse discharges from pharmaceutical facilities, higher mass loads of DC-AZI
367 were determined in RW than in SE. As pointed out above, DC-AZI can have multiple sources,
368 including AZI instability in pharmaceutical formulations, human metabolism and additional formation
369 in the sewer system. A high percentage of DC-AZI in RW (17%) indicated the prevalence of the last
370 two mechanisms. Our previous aerobic biodegradation study³¹ suggested the loss of cladinose as an
371 important step in the overall transformation pathway, but did not show any accumulation of DC-AZI
372 as a major stable TP. This is consistent with the observations on DC-AZI levels in SE over the one-year
373 cycle. Since kinetics of both abiotic and biotic transformations are expected to depend on
374 environmental factors such as water temperature, pH and microbial community composition, further
375 studies are needed to address this issue in more detail.

376 It is important to point out that, as a combined result of high inputs in RW and low removal during
377 conventional wastewater treatment, substantial amount (up to 8800 g/day) of AZI-related
378 compounds is discharged from the investigated WWTP into the receiving surface water. Due to the
379 significant contribution of industrial inputs, the population normalized average mass load of AZI-
380 related compounds was 3.3 g/day/1000 inhabitants, which is significantly higher than the average
381 mass loads of macrolide antibiotics in municipal WWTPs reported in some previous studies^{19,44} and
382 the mass loads which can be estimated from the consumption data (0.48 g/day/1000 inhabitants).³³

383 Finally, it should be noted that this study was focused only on the dissolved phase. For the complete
384 mass balance study in the wastewater treatment, the analysis of macrolide compounds incorporated
385 into the suspended particles should be performed as well. This might be especially important for AZI-
386 related compounds, due to the strong sorption properties of parent AZI.⁵

387

388 ***3.4. Screening for additional transformation products***

389 In contrast to synthesis by-products and human metabolites, the term transformation product in this
390 work was meant for the macrolide products formed primarily in the wastewater itself. Wastewater
391 extracts prepared for the target analyses were also screened for additional AZI- and CLA-related
392 compounds using high resolution MS in combination with MRM (MS/MS) confirmation. The
393 emphasis was on TPs which were previously identified in model aerobic biotransformation
394 experiments using enriched activated sludge culture from the same WWTP.³¹ For structural
395 assignments see SI (Fig S2 and S3). It should be pointed out that pure reference standards of these
396 compounds were not available, which prevented obtaining fully quantitative results. Nevertheless,
397 the media from biotransformation experiments described in Terzic et al. (2018)³¹ were applied as
398 qualitative standards, providing link to structural information, as well as to retention time data for
399 the additional TPs. The overview of the TPs encompassed by this screening is presented in Table 3.
400 The accurate mass data for all detectable TPs were within the tolerance limits of 5 mDa, while
401 additional confirmation was achieved by MRM analysis, using two characteristic transitions for each
402 TP (Table S3, SI). The most abundant additional AZI TPs were those which included macrolide ring
403 opening and/or deglycosylation (loss of sugar moieties; TP 767, TP 610, TP 452, TP 592), followed by
404 oxidative transformations of the linearized macrolide TPs (TP 376, TP 374bc, TP 358).

405 Semi-quantitative estimates, assuming equal molar responses for all AZI-related compounds in QToF
406 analysis, indicated that the concentrations of individual TPs were always less than 5% of AZI
407 concentration (average about 1%). However, their relative contribution was significantly enhanced in

408 SE as compared to RW. The increase during secondary treatment varied from 3 for TP 394 to 2000
409 times for TP 376a (Figure S7, SI), supporting the hypothesis that they can be regarded as highly
410 specific markers of aerobic transformations, although their contribution to the overall mass balance
411 during secondary treatment was rather low.

412 Regarding additional TPs of CLA, three prominent TPs were identified. The most abundant one was
413 CLA-OH, which represented approximately 50% of the parent CLA levels (obtained by assuming equal
414 responses of CLA-OH and CLA) and very probably contributed significantly to the CLA mass balance.
415 The preliminary evidence does not indicate its formation during secondary treatment, however this
416 compound remains highly relevant for the overall risk assessment, because it fully retains the
417 antibiotic activity of CLA. In contrast to CLA-OH, concentration of phosphorylated CLA was about 5.9
418 times higher in SE than in RW, indicating net formation during secondary treatment. The similar
419 SE/RW ratio was observed for TP 766, which is formed by opening of the CLA macrolide ring,
420 indicating similarity with transformation pattern of AZI.

421 Collectively, in the present study we demonstrated for the first time the need for a more
422 comprehensive approach when addressing the issue of macrolide antibiotics as contaminants of
423 emerging concern. Complex characterization of macrolide-derived compounds, including
424 determination of synthesis by-products, human metabolites and TPs, provides a basis for decoupling
425 of industrial and therapeutic sources from the *in-situ* transformations. The overall removal of
426 macrolide-derived compounds during the conventional wastewater treatment was shown to be
427 rather low, which warrants a careful ecotoxicological evaluation of their complex mixtures present in
428 municipal sewage effluents and calls urgently for a more detailed understanding of their behavior
429 and fate in the aquatic environment. Finally, the results obtained in this study could also contribute
430 to better understanding of issues related to spreading of antimicrobial resistance in the environment.

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436

437 ASSOCIATED CONTENT**438 Supporting Information**

439 Structures and abbreviations of target compounds; Key characteristics of the investigated WWTP;
440 Brief description of analytical procedure and method validation data; MRM transitions used for the
441 analyses of additional AZI and CLA TPs; Structures of additional AZI and CLA TPs included in suspect
442 screening; Annual variability of daily mass loads of target compounds in RW and SE; Seasonal trends
443 of AZI and CLA in RW; Ratios of additional AZI and CLA TPs in SE and RW samples. This information is
444 available free of charge via the Internet at <http://pubs.acs.org>.

445

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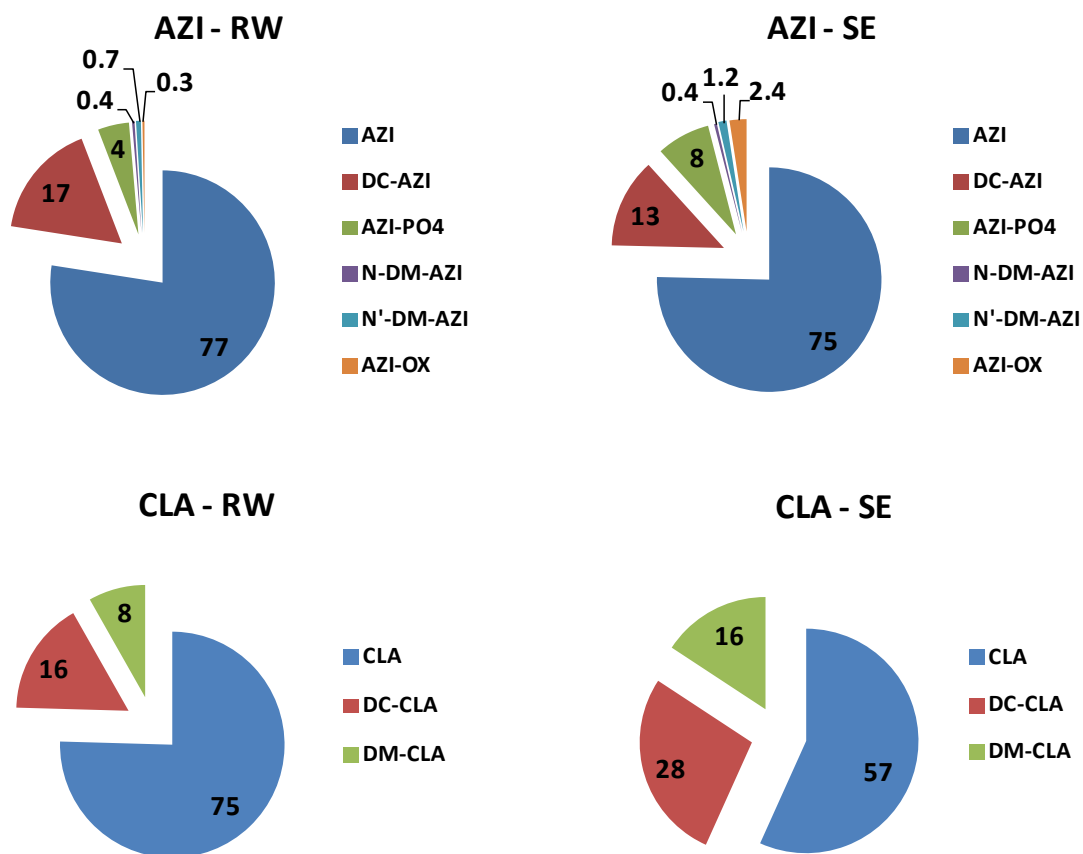
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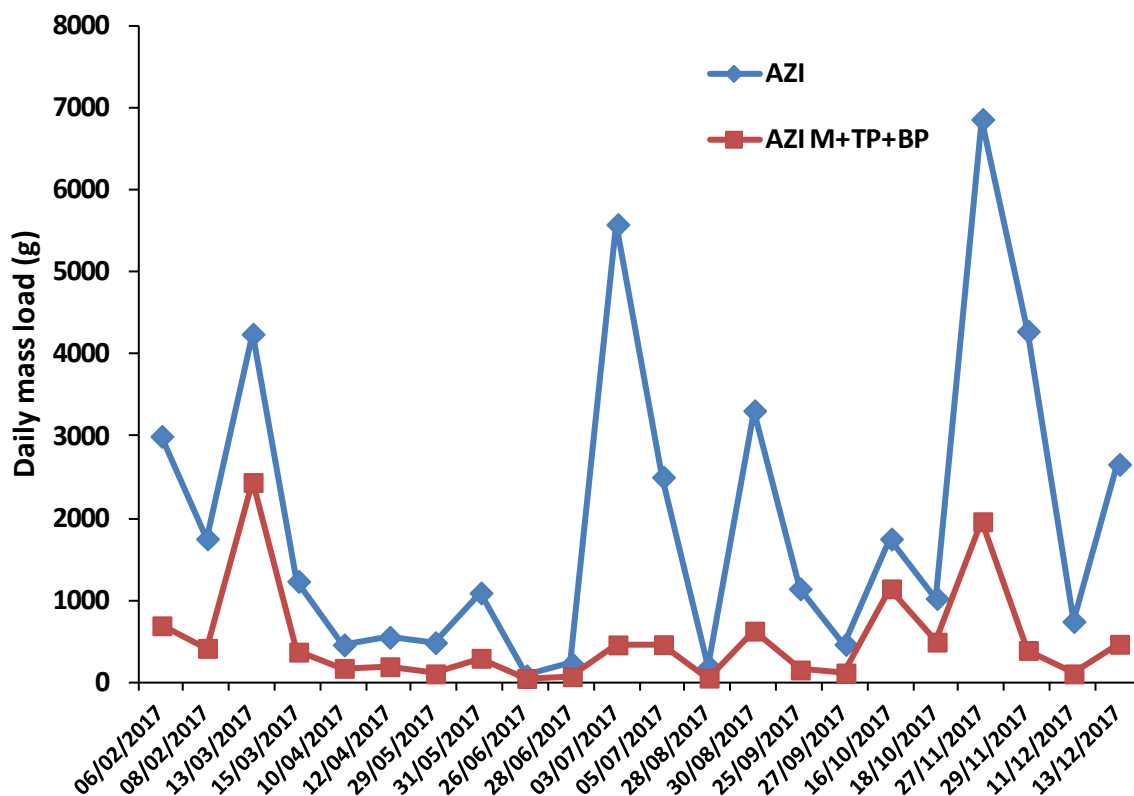
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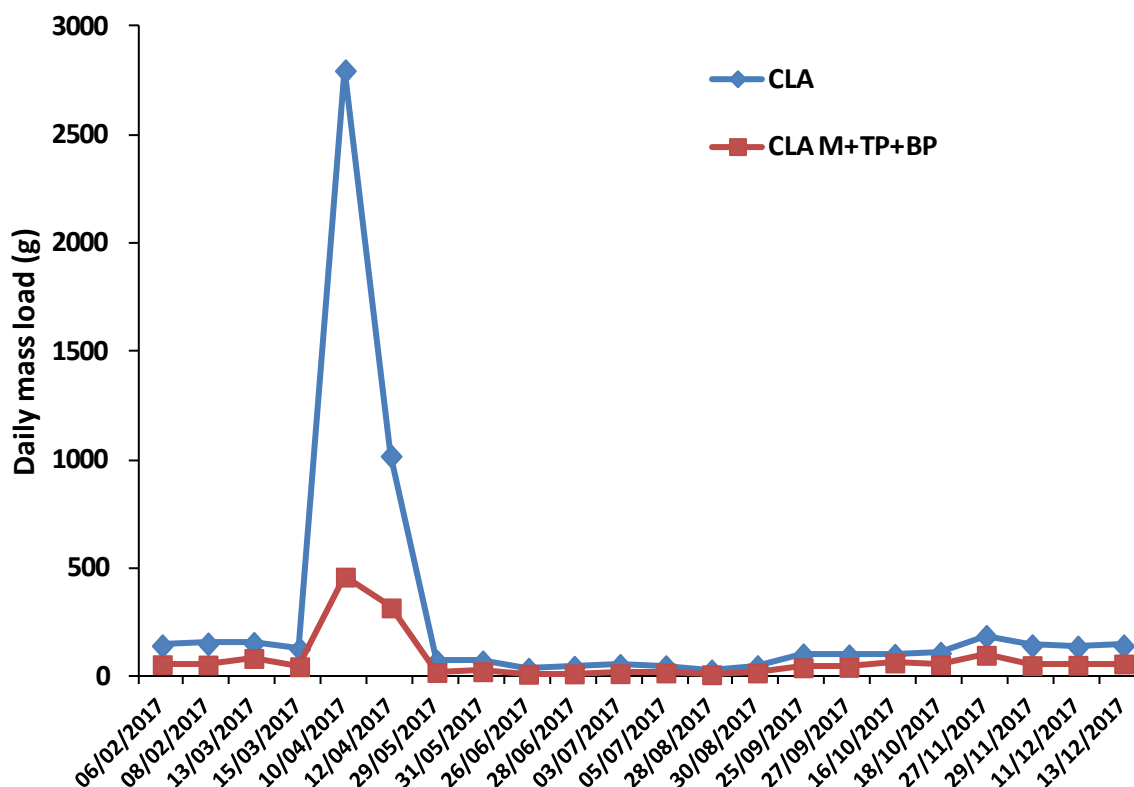


578

579 **Figure 1.** Average relative contribution of individual compounds to the total loads of azithromycin
 580 (AZI) and clarithromycin (CLA) in raw wastewater (RW) and secondary effluent (SE) of the wastewater
 581 treatment plant of the city of Zagreb.

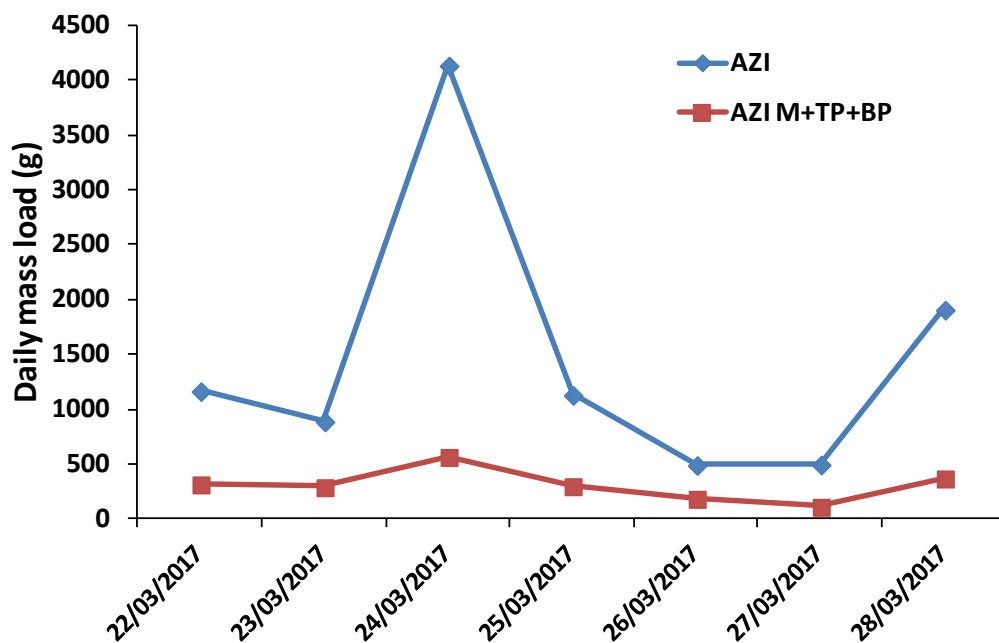


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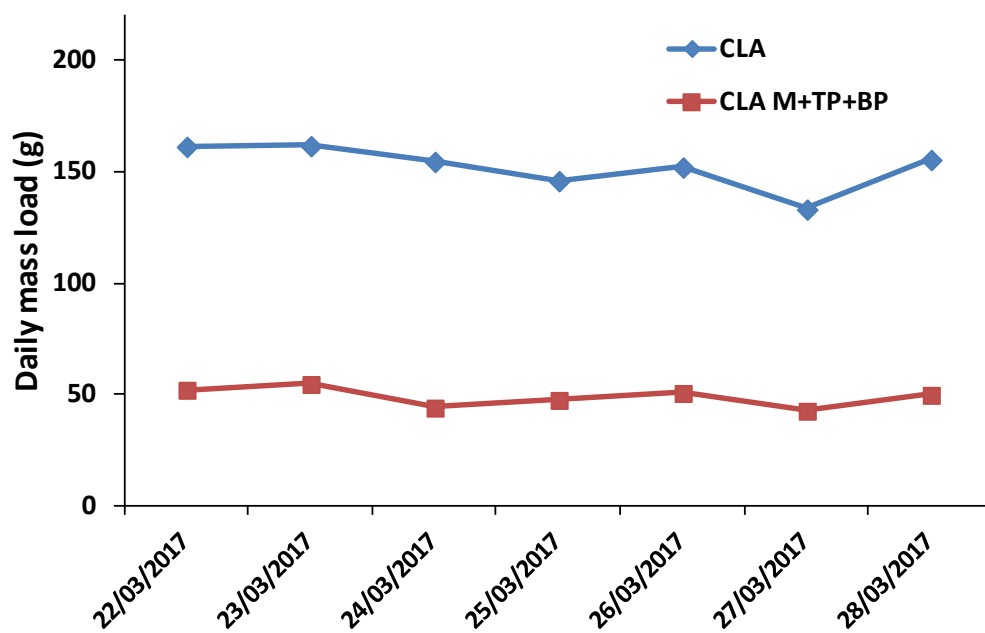


583

584 **Figure 2.** Daily mass loads of azithromycin (AZI) and clarithromycin (CLA), along with the sum of their
 585 metabolites (M), transformation products (TP) and by-products (BP), to the wastewater treatment
 586 plant of the city of Zagreb during 2017 (one-year sampling interval).



587



588

589 **Figure 3.** Daily mass loads of azithromycin (AZI) and clarithromycin (CLA), along with the sum of their
 590 metabolites (M), transformation products (TP) and by-products (BP), to the wastewater treatment
 591 plant of the city of Zagreb during the one-week sampling interval.

592 **Table 1.** Occurrence of target compounds in the wastewater treatment plant of the city of Zagreb.

Compound	Raw wastewater (<i>n</i> =29)					Secondary effluent (<i>n</i> =29)				
	Concentration (ng/L)				FD (%)	Concentration (ng/L)				FD (%)
	Minimum	Maximum	Average ± SD	Median		Minimum	Maximum	Average ± SD	Median	
AZI	269	22730	5837 ± 5307	4021	100	316	20178	5453 ± 4816	4451	100
DC-AZI	87	7522	992 ± 1485	486	100	105	2370	736 ± 546	623	100
AZI-PO ₄	49	1171	374 ± 275	311	100	73	2209	618 ± 518	418	100
N-DM-AZI	<MQL	74	34 ± 19	32	79	<MQL	70	38 ± 20	43	76
N'-DM-AZI	10*	97	52 ± 24	57	100	13	171	83 ± 43	74	100
AZI-OX	<MQL	83	24 ± 24	12	93	16	479	180 ± 146	116	100
ERY-H ₂ O	10*	129	26 ± 23	21	97	4.2*	75	19 ± 14	16	100
ERY-EE	3.3*	187	22 ± 38	13	100	<MQL	35	8.5 ± 8.0	6.1	59
DM-ERY	<MQL	<MQL	–	–	0	<MQL	<MQL	–	–	0
ERY-IE	<MQL	<MQL	–	–	0	<MQL	<MQL	–	–	0
ERY-OX	<MQL	12	7.0* ± 2.3	6.2	21	5.5	12	7.5 ± 2.5	6.6	24
CLA	111	10491	828 ± 1961	389	100	25	2517	266 ± 461	178	100
DC-CLA	13	1559	142 ± 328	56	100	11	1570	102 ± 284	44	100
DM-CLA	20	170	88 ± 35	100	100	18	153	72 ± 30	72	100
ROX	<MQL	<MQL	–	–	0	<MQL	<MQL	–	–	0

593 FD - Frequency of detection

594 *The concentrations lower than method quantification limit (MQL), but above method detection limit

595 **Table 2.** Total mass loads and average removal of target compounds in the wastewater treatment
 596 plant of the city of Zagreb during the one-week sampling interval.

Compound	Total mass load (g)		Average removal (%)
	Raw wastewater	Secondary effluent	
AZI	10249	10547	-3
DC-AZI	918	2400	-162
AZI-PO ₄	1150	1238	-8
N'-DM-AZI	69	184	-167
AZI-OX	20	562	-2659
ERY-H ₂ O	98	64	34
ERY-EE	115	20	83
CLA	1064	410	61
DC-CLA	128	122	5
DM-CLA	215	130	40

597

598 **Table 3.** Additional azithromycin (AZI) and clarithromycin (CLA) transformation products (TPs)
 599 recently identified in model biotransformation experiments²³, in raw wastewater (RW) and
 600 secondary effluent (SE) from the wastewater treatment plant of the city of Zagreb.

Compound	ELEMENTAL COMPOSITION /[M+H]	<i>m/z</i> (theoretical)	QToF ¹			QqQ ²		
			Retention time (min)	<i>m/z</i> (experimental)	Difference (mDa)	2 specific MRM transitions	Detection frequency (%)	
							RW	SE
AZI TP 394	C ₁₉ H ₄₀ NO ₇	394.2805	2.5	ND	NA	+	28	10
AZI TP 452	C ₂₂ H ₄₆ NO ₈	452.3223	2.6	452.3225	+0.2	+	0	38
AZI TP 376a	C ₁₉ H ₃₈ NO ₆	376.2699	2.6	376.2685	-1.4	+	28	100
AZI TP 376b	C ₁₉ H ₃₈ NO ₆	376.2699	2.7	376.2684	-1.5	+	24	97
AZI TP 392	C ₁₉ H ₃₈ NO ₇	392.2648	2.8	ND	NA	+	0	14
AZI TP 374a	C ₁₉ H ₃₆ NO ₆	374.2543	2.8	374.2532	-1.1	+	0	100
AZI TP 374b	C ₁₉ H ₃₆ NO ₆	374.2543	2.9	374.2533	-1.0	+	41	100
AZI TP 374c	C ₁₉ H ₃₆ NO ₆	374.2543	3.0	374.2533	-1.0	+	69	100
AZI TP 450	C ₂₂ H ₄₄ NO ₈	450.3067	3.0	ND	NA	+	3	7
AZI TP 358	C ₁₉ H ₃₆ NO ₅	358.2593	3.3	ND	NA	+	52	86
AZI TP 356	C ₁₉ H ₃₄ NO ₅	356.2434	3.6	ND	NA	+	0	52
AZI TP 767	C ₃₈ H ₇₅ N ₂ O ₁₃	767.5269	3.7	ND	NA	+	79	79
AZI TP 610	C ₃₀ H ₆₀ NO ₁₁	610.4166	4.3	610.4172	+0.6	+	28	100
AZI TP 608	C ₃₀ H ₅₈ NO ₁₁	608.4010	4.6	ND	NA	+	7	34
AZI TP 592	C ₃₀ H ₅₈ NO ₁₀	592.4061	5.6	592.4046	-1.5	+	97	100
CLA TP 766	C ₃₈ H ₇₂ NO ₁₄	766.4953	4.9	ND	NA	+	34	69
CLA TP 764a ³	C ₃₈ H ₇₀ NO ₁₄	764.4796	5.2	764.4831	+3.5	+	100	100
CLA TP 828 ⁴	C ₃₈ H ₇₁ NO ₁₆ P	828.4510	5.9	828.4525	+1.5	+	97	100
CLA TP 764b ⁵	C ₃₈ H ₇₀ NO ₁₄	764.4796	6.9	ND	NA	+	100	100

601 ¹QToF data for AZI TPs AND CLA TPs comprise the data obtained for SE sample collected on 31st May 2017 and
 602 10th April 2017, respectively; ²Confirmation obtained in at least one sample by MRM analysis using QqQ MS;
 603 ³CLA 764a = CLA-OH; ⁴CLA 828 = CLA-PO₄; ⁵CLA 764b = CLA-*N*-oxide; QToF – quadrupole time-of-flight mass
 604 spectrometer; QqQ – triple quadrupole mass spectrometer; ND – not detected