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Biotransformation of macrolide antibiotics using enriched activated sludge culture: Kinetics, transformation routes and ecotoxicological evaluation

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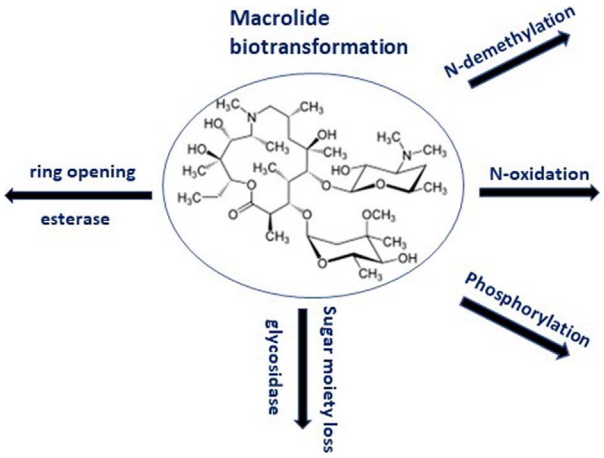
GRAPHICAL ABSTRACT



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ABSTRACT

The biotransformation of three prominent macrolide antibiotics (azithromycin, clarithromycin and erythromycin) by an activated sludge culture, which was adapted to high concentrations of azithromycin (10 mg/L) was investigated. The study included determination of removal kinetics of the parent compounds, identification of their major biotransformation products (TPs) and assessment of ecotoxicological effects of biotransformation. The chemical analyses were performed by ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry, which enabled a tentative identification of TPs formed during the experiments. The ecotoxicological evaluation included two end-points, residual antibiotic activity and toxicity to freshwater algae. The enriched activated sludge culture was capable of degrading all studied macrolide compounds with high removal efficiencies (> 99%) of the parent compounds at elevated concentrations (10 mg/L). The elimination of all three macrolide antibiotics was associated with the formation of different TPs, including several novel compounds previously unreported in the literature. Some of the TPs were rather abundant and contributed significantly to the overall mass balance at the end of the biodegradation experiments. Biodegradation of all investigated macrolides was associated with a pronounced reduction of the residual antibiotic activity and algal

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toxicity, indicating a rather positive ecotoxicological outcome of the biotransformation processes achieved by the enriched sludge culture.

1. Introduction

Macrolide antibiotics are a numerous class of natural and semi-synthetic antimicrobial compounds, which are widely used to treat respiratory tract and soft-tissue infections. The typical macrolide antibiotics are relatively large molecules, which consist a macrocyclic lactone ring containing 14–16 atoms, substituted with hydroxyl, alkyl and ketone groups and with neutral or amino sugars bound to the ring by substitution of hydroxyl groups. The most commonly used modern macrolide antibiotics are semisynthetic derivatives of erythromycin (ERY), which possess significantly improved clinical properties in terms of antimicrobial activity, stability, bioavailability and pharmacokinetics [1].

After therapeutic use in human and veterinary medicine, a large percentage of the applied macrolide dose is released from the body unchanged [2], resulting in significant loads of the parent macrolides in municipal wastewaters and farm effluents. Consequently, a number of literature reports demonstrated their widespread occurrence in municipal and industrial wastewaters and ambient waters [3–5], raising concerns about the possible selection for and spread of antibiotic resistance in the aquatic environment [6]. Moreover, studies of the behaviour of macrolide antibiotics in conventional activated sludge treatment indicated that their removal is incomplete, which may lead to comparatively high exposure concentrations in receiving ambient waters [7–9].

One of the possible strategies to limit proliferation of resistant bacteria is to reduce the exposure to antimicrobials by improving their removal from wastewater [10]. The best way to achieve this goal would be through efficient biotransformation, preferably mineralization to carbon dioxide and inorganic salts, or through some alternative abiotic transformation process such as ozonation [11] and photocatalytic degradation [12]. Transformation processes, however, may often be ineffective or even ecotoxicologically questionable when the transformation of the parent compounds leads to the formation of various stable products whose effects in the environment are largely unknown [13]. It was shown that photolytic removal of different types of macrolide antibiotics was accompanied by the formation of a large variety of transformation products [14,15]. In contrast, little is known about their biodegradability, possible biotransformation products and biotransformation pathways. Whereas some recent studies indicated that ERY was efficiently biotransformed both under aerobic and anaerobic conditions [16], available reports on the behaviour of macrolide antibiotics in conventional WWTPs and membrane bioreactors [5,17,18] suggested that their biodegradation was incomplete. Several biotransformation products of CLA and AZI have been identified in real municipal and industrial wastewater systems [9,19], however none of these studies investigated the transformation processes in more detail. Our earlier study on elimination of azithromycin and roxitromycin in a membrane bioreactor [20] showed that their biological removal was incomplete and indicated significant formation of the corresponding phosphorylated transformation products [21]. Since phosphorylation is a well-known microbial strategy for the inactivation of macrolide antibiotics [22], this finding indicated that the existing enzymatic mechanisms of macrolide resistance might play an important role in the biotransformation pathways of macrolides.

The aim of the present study was to investigate the ability of the activated sludge culture adapted to high concentration of AZI (10 mg/L) to degrade three prominent representatives of macrolide antibiotics (AZI, CLA and ERY) under aerobic conditions. These macrolides have been recently selected for inclusion in the EU Watch list [23], which warrants a comprehensive environmental assessment of these compounds, including the role of their TPs. This study included the production of an active enrichment culture, determination of the removal kinetics of the parent compounds at elevated concentrations typical of industrial wastewaters, identification of transformation products and ecotoxicological evaluation of biotransformation using two different end-points. Based on identified TPs, tentative biotransformation routes involved in the elimination of AZI have been proposed.

2. Materials and methods

2.1. Chemicals and reagents

Azithromycin (AZI) (purity > 95%) was purchased from Fluka (Buchs, Switzerland). Erythromycin (ERY) (purity 98%) and clarithromycin (CLA) (purity > 95%) were obtained from Sigma-Aldrich (Steinheim, Germany). Phosphorylated macrolides (AZI TP (829), CLA TP (828) and ERY-TP (814)) and 14-OH-CLA (CLA TP (764a)) were prepared as described by Senta et al. [9]. All other reference materials used for the confirmation of identified TPs were supplied by Toronto Research Chemicals (Canada). The purity of the reference materials used for confirmatory purposes was ≥98%. Ammonium chloride (purity > 99.5%) and ammonium nitrate (purity > 99.5%) were purchased from Gram-mol (Zagreb, Croatia). Formic acid (LC-MS grade) and ammonium formate (purity ≥ 99%) were purchased from Sigma-Aldrich. All other chemicals used for biodegradation media were of analytical grade purity and supplied by Kemika (Zagreb, Croatia). R2A broth (Lab M Limited, UK) supplemented with 1.5% agar (Biolife, Milan, Italy) was used to prepare R2A agar plates. LC–MS grade solvents (acetonitrile and methanol) were products of J.T. Baker (Deventer, the Netherlands). Mueller-Hinton agar and Mueller-Hinton broth were supplied by Sigma-Aldrich. Ultrapure water was produced using an Elix-Milli-Q system (Millipore, Bedford, MA, USA).

Solid-phase extraction (SPE) cartridges Oasis HLB (60 mg/3 mL) were supplied by Waters (Milford, MA, USA). The individual stock solutions (10 mg/mL and 1 mg/mL) of macrolide compounds were prepared in LC–MS grade methanol. The reference standards used for the qualitative and quantitative LC–MS analyses were prepared in 50 mM ammonium formate in the concentration range of 0.01–2.5 μg/mL.

2.2. Enrichment of azithromycin-degrading activated sludge culture

Activated sludge was originally collected from the aeration tank of the Central wastewater treatment plant of the city of Zagreb, Croatia (MLSS concentration of 3.5 g/L). Ten milliliters of fresh activated sludge were transferred into a 300-mL Erlenmeyer flask containing 90 mL of modified mineral salt medium [24]. Modification was made by adding a high concentration of AZI (10 mg/L), 1 g/L of glucose, 50 mg/L of yeast extract (AMM) and either 100 mg/L NH_4Cl or 75 mg/L NH_4NO_3 in the medium. AZI was added from the stock solution prepared in methanol (10 mg/mL). The flasks were incubated in the dark at 28 °C on a rotary shaker operated at 180 rpm. Every two weeks, 5% of enriched culture was transferred to a fresh medium (50 mL) and incubated under the same conditions. After 4 months of enrichment (total of 8 culture transfers), biomass was centrifuged (9279 g, 15 min), resuspended in physiological saline (0.85% NaCl) and stored at −20 °C in glycerol (16% *v/v* as the final concentration).

2.3. Azithromycin biotransformation and culture growth

2.3.1. Inoculum preparation

In preliminary AZI biotransformation experiments the inocula were prepared by centrifuging (9279 g, 15 min, 4 °C) the fresh cultures from the fourth and eighth transfer and suspending the cells in physiological saline (0.85% NaCl). Cell numbers were quantified by plating on R2A plates. For macrolide biotransformation experiments, frozen enrichment from the eighth transfer, initially cultured in NH_4Cl-containing AMM, was grown in the mineral salt medium (MM) supplemented with macrolide (10 mg/L) as the sole organic C source and NH_4Cl (100 mg/L) as the sole N source on a rotary shaker at 28 °C for 1 week. The cells were harvested by centrifugation (9279 g, 15 min), washed twice and resuspended in physiological saline to give a density of approximately 10^9 colony forming units (CFU)/mL.

2.3.2. Preliminary AZI biodegradation tests

Preliminary biotransformation experiments were conducted to test the ability of enrichments to degrade AZI. These experiments were performed in shake-flasks in the dark at 28 °C after an enrichment time of 2 and 4 months. Cells from enrichment cultures initially cultured in NH_4Cl-containing AMM were used as inocula for 100-mL AMM with NH_4Cl and/or 100 mL MM with AZI as the sole organic C source and NH_4Cl as the sole N source. Fresh enrichments initially cultured in NH_4NO_3-containing AMM were used as inocula for 100-mL AMM supplemented with NH_4NO_3, either 75 or 200 mg/L. Liquid samples (1.5 mL) were collected periodically to monitor the change in AZI concentration and possible formation of transformation products. Uninoculated flasks were used as abiotic controls and flasks inoculated with autoclaved culture as adsorption controls.

2.3.3. Macrolide antibiotic biodegradation tests

Triplicate flasks (300 mL) containing MM (110 mL) with individual macrolide antibiotic (AZI, ERY or CLA, each 10 mg/L) as the sole organic C source and NH_4Cl as the sole N source were inoculated with acclimated sludge to give an initial cell density of approx. 5×10^8 CFU/mL. The cultures were incubated at 28 °C on a rotary shaker at 180 rpm for 12 days. Abiotic and adsorption controls were prepared as well. Aliquots (1.5 mL) for LC–MS analyses as well as for toxicity evaluation were withdrawn periodically, centrifuged immediately (9279*g*, 10 min), and kept frozen (−20 °C) in HDPP vials until analyses. Culture growth was monitored by plating appropriate dilutions on R2A plates.

2.3.4. Kinetics analysis

The degradation kinetics was modelled by either first-order kinetics or by a logistic model.

Degradation rate constants for both models were estimated by performing a nonlinear least squares regression analysis. Goodness of fit was assessed using the fitting value R^2. Analyses were performed using Statistica Version 10.0 (StatSoft Inc., Tulsa, USA) at a $p < .05$ significance level. The details of the kinetic analysis are given in Electronic Supplementary Material.

2.4. Analyses of macrolide antibiotics and their transformation products

Before LC–MS analyses the samples were diluted 5 times in 50 mM ammonium formate. The analysis of the macrolide antibiotics was performed using ultrahigh-performance liquid chromatography (UPLC) coupled to quadrupole-time-of-flight mass spectrometry (QTOFMS). UPLC separation was performed using a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) equipped with a binary solvent delivery system and autosampler. The chromatographic separations employed a column (50 mm × 2.1 mm) filled with a 1.7 µm BEH C_{18} stationary phase (Waters Corp., Milford, MA, USA). Binary gradients at a flow rate of 0.4 mL/min were applied for the elution. In the positive ionization (PI) mode the eluents A and B were 0.1% HCOOH in water and 0.1% HCOOH in acetonitrile, respectively. The eluents used in the negative ionization (NI) mode consisted of (A) water and (B) acetonitrile without addition of formic acid. The analyses in both polarity modes were performed by applying the following gradient: the elution started at 5% B and after a 1 min of isocratic hold, the percentage of B was linearly increased to 50% in 8 min.

The mass spectrometry was performed on a QTOF Premier instrument (Waters Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray interface. The drying gas and nebulizing gas was nitrogen, whereas argon was used as the collision gas in MS–MS experiments. The desolvation gas flow was set to 700 L/h at a temperature of 300 °C. The cone gas flow was adjusted to 25 L/h, and the source temperature to 120 °C. The capillary voltages in the PI and NI mode were 3500 V and 3000 V, respectively, whereas the cone voltage in both modes was set to 30 V. The MS data were collected between m/z 50–1000, applying a collision energy of 4 eV.

All spectra were recorded using the extended dynamic range (DRE) option to correct for possible peak saturations, and the data were collected in the centroid mode with a scan time of 0.08 s and interscan time of 0.02 s. To ensure maximum accuracy and reproducibility of the system, all acquisitions were performed using an independent reference spray via the lock spray interface. Leucine enkephaline was applied as a reference mass both in PI and NI mode.

The data were processed using the MassLynx software incorporated in the instrument. The quantification of the parent compounds was performed by using the external calibration curves.

2.5. Antibiotic activity evaluation

An antibiotic activity test based on the inhibition of bacterial growth was performed according to Dodd et al. [11] with slight modifications (see Electronic Supplementary Material). Briefly, *Bacillus subtilis* Marburg, ATCC 6051 culture, was seeded on Mueller-Hinton agar plates, and the culture was grown in sterile conditions at 30 °C. Starter broth culture was prepared by suspending *B. subtilis* colonies in Mueller-Hinton broth and growing over night at 30 °C on an agitation

Table 1
Kinetic parameters of azithromycin biodegradation after 2 and 4 months of enrichment under different testing conditions.

Enrichment time	Testing medium	N (mg L^{-1})	Kinetic parameters			
			k (d^{-1})	$t_{1/2}$ (d)	R^2	μ_{max} (day^{-1})
2 months	AZI + GLU + YE + NH_4Cl	22	0.147	4.71	0.92	nd
	AZI + GLU + YE + NH_4NO_3	26	0.148	4.68	0.80	nd
	AZI + GLU + YE + NH_4NO_3	70	–	–	–	nd
4 months	AZI + GLU + YE + NH_4NO_3	26	0.214	3.24	0.89	nd
	AZI + GLU + YE + NH_4Cl	22	0.243	2.85	0.95	0.446
	AZI + NH_4Cl	22	0.303	2.29	0.96	0.291

N – nitrogen; AZI – azithromycin; GLU – glucose; YE – yeast extract; k – kinetic degradation rate constant; $t_{1/2}$ – degradation half-life; R^2 – correlation coefficient; – no observed degradation; nd – not determined; AZI concentration 10 mg/L; glucose concentration 1 g/L; kinetic parameters calculated using a logistic model.

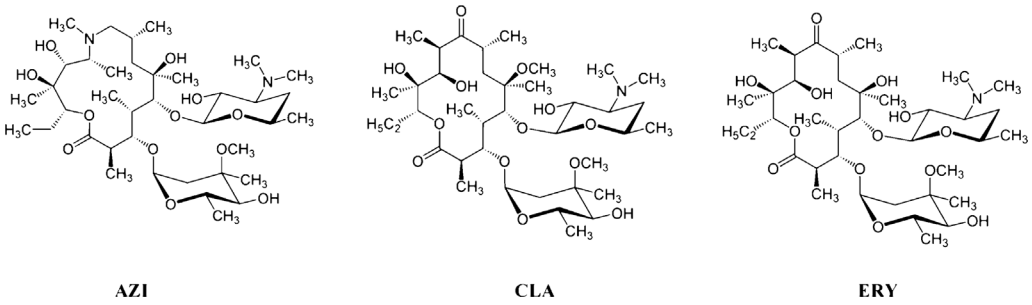

Fig. 1. Chemical structures of azithromycin (AZI), clarithromycin (CLA) and erythromycin (ERY).

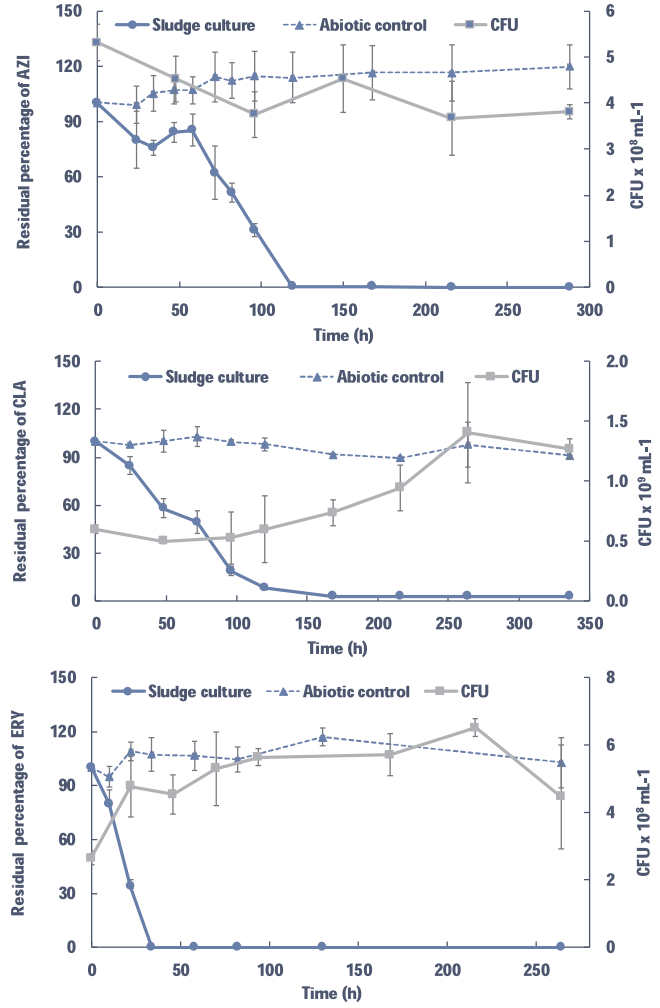

Fig. 2. Removal kinetics of azithromycin (AZI), clarithromycin (CLA) and erythromycin (ERY) and changes in number of colony forming units (CFU) in biodegradation experiments performed by the enriched sludge culture.

plate with rotation at 250 rpm.

Antibiotic activity of the samples was determined by observing the growth inhibition of the bacterial culture by measuring absorbance at a wavelength of 625 nm as an equivalent of the bacterial cell density.

2.6. Chronic toxicity

2.6.1. Sample preparation

To eliminate the salts contained in the medium used for biodegradation studies, the samples for the evaluation of algal toxicity were previously percolated through Oasis HLB columns. Half a milliliter of the sample was percolated through the extraction cartridges previously preconditioned with 3 mL of methanol, ultrapure water and spring water. After the sample enrichment, the residual salts were washed out from the cartridge with 3 mL of ultrapure water and discarded, while the adsorbed antibiotics were eluted with 2 mL of methanol by applying a gravity flow. The methanol was evaporated, and the dry residue was re-dissolved in 0.5 mL of the ISO/FDIS 8692 culture medium.

2.6.2. Chronic toxicity evaluation

Chronic toxicity of antibiotic samples was evaluated using the freshwater green algae *Desmodesmus subspicatus* (86.81 SAG) grown in ISO/FDIS 8692 culture medium, as described in detail in ISO [25]. The test was conducted in 96 microwell plates as described previously [26,27] with slight modifications (for details see Supplementary Material). The average specific growth rate was calculated and subsequently used to calculate the inhibition and then fitted to a three-parameter sigmoid dose–response equation. The dose–response curve of $K_2Cr_2O_7$ was included as a reference standard in all experiments.

3. Results and discussion

3.1. Adaptation of activated sludge to azithromycin

The initial activated sludge culture, obtained from the WWTP of the city of Zagreb, was unable to degrade AZI at high concentrations (1–10 mg/L), which was in agreement with its relatively low removal in this WWTP [5]. During the adaptation period of 4 months, the performance of the microcosm enrichments was tested two times. After an adaptation time of 2 months under cometabolic conditions with

Table 2
Kinetic parameters of the degradation of macrolides added as the sole organic carbon source.

Macrolide	Kinetic model	Kinetic parameters			
		k (d^{-1})	$t_{1/2}$ (d)	R^2	μ_{max} (d^{-1})[b]
Azithromycin	Logistic	1.656 ± 0.216	3.42 ± 0.05	0.95	–[a]
Erythromycin	First-order	1.224 ± 0.6	0.57 ± 0.277	0.95	0.624 ± 0.168
Clarithromycin	First-order	0.323 ± 0.105	2.14 ± 0.7	0.96	0.525 ± 0.298

k-degradation rate constant; $t_{1/2}$ – degradation half-life; R^2 – correlation coefficient.
Data are reported as mean ± standard deviation. The results were considered statistically significant at $p < .05$.
[a] Growth inhibition.
[b] μ_{max}–maximum specific growth rate, estimated by finite difference method.

Table 3
List of biotransformation products (TPs) of azithromycin (AZI), erythromycin (ERY) and clarithromycin (CLA) identified by UPLC-QTOF/MS analyses in positive ionization mode (PI) during the degradation experiments performed by the enriched mixed microbial culture.

RT (min)	Compound	*m/z* (experimental)	Elemental Composition/[M+H]	*m/z* (theoretical)	difference (mDa)	abundance
AZITHROMYCIN						
4.5	AZI	749.5151	C38H73N2O12	749.5164	−1.3	NA
BIOTRANSFORMATION PRODUCTS OF AZI						
2.5	AZI TP (394)	394.2791	C19H40NO7	394.2805	−1.4	+ +
2.6	AZI TP (452)	452.3217	C22H46NO8	452.3223	−0.6	+ + +
2.6	AZI TP (376a)	376.2676	C19H38NO6	376.2699	−2.3	+ +
2.7	AZI TP (376b)	376.2711	C19H38NO6	376.2699	+1.2	+ + +
2.8	AZI TP (392)	392.2647	C19H38NO7	392.2648	−0.1	+ +
2.8	AZI TP (374a)	374.2530	C19H36NO6	374.2543	−1.3	+ +
2.9	AZI TP (374b)	374.2521	C19H36NO6	374.2543	−2.2	TRACE
3.0	AZI TP (374c)	374.2527	C19H36NO6	374.2543	−1.6	+ + +
3.0	AZI TP (450)	450.3076	C22H44NO8	450.3067	+0.9	+ +
3.3	AZI TP (358)	358.2580	C19H36NO5	358.2593	−1.3	TRACE
3.6	AZI TP (356)	356.2438	C19H34NO5	356.2434	+0.4	TRACE
3.7	AZI TP (434)	434.3143	C22H44NO7	434.3118	+2.5	TRACE
3.7	AZI TP (767)	767.5267	C38H75N2O13	767.5269	−0.2	TRACE
4.0	AZI TP (765a)	765.5123	C38H73N2O13	765.5113	+1.0	TRACE
4.3	AZI TP (610)	610.4167	C30H60NO11	610.4166	+0.1	+ + +
4.3	AZI TP (829)	829.4844	C38H74N2O15P	829.4827	+1.7	TRACE
4.4	AZI TP (735)	735.5044	C37H71N2O12	735.5009	+3.5	TRACE
4.6	AZI TP (608)	608.4003	C30H58NO11	608.4010	−0.7	TRACE
4.8	AZI TP (765b)	765.5125	C38H73N2O13	765.5113	+1.2	TRACE
5.6	AZI TP (592)	592.4054	C30H58NO10	592.4061	−0.7	TRACE
ERYTHROMYCIN						
5.6	ERY	734.4703	C37H68NO13	734.4691	+1.2	NA
BIOTRANSFORMATION PRODUCTS OF ERY						
4.2	ERY TP (750a)	750.4668	C37H68NO14	750.4640	+2.8	TRACE
4.9	ERY TP (814)	814.4375	C37H69NO16P	814.4354	+2.1	TRACE
5.1	ERY TP (576)	576.3757	C29H54NO10	576.3748	+0.9	+ +
5.5	ERY TP (720)	720.4555	C36H66NO13	720.4534	+2.1	TRACE
6.0	ERY TP (750b)	750.4653	C37H68NO14	750.4640	+1.3	TRACE
6.2	ERY TP (419)	419.2621	C21H39O8	419.2645	−2.4	+ + +
CLARITHROMYCIN						
6.6	CLA	748.4846	C38H70NO13	748.4847	−0.1	NA
BIOTRANSFORMATION PRODUCT OF CLA						
3.8	CLA TP (590)	590.3908	C30H56NO10	590.3904	+0.4	TRACE
4.9	CLA-TP (766)	766.4949	C38H72NO14	766.4953	−0.4	+ +
5.2	CLA-TP (764a)	764.4816	C38H70NO14	764.4796	+2.0	TRACE
5.9	CLA TP (828)	828.4498	C38H71NO16P	828.4510	−1.2	+ + +
6.4	CLA TP (734)	734.4702	C37H68NO13	734.4691	+2.9	TRACE
6.9	CLA TP (764b)	764.4793	C38H70NO14	764.4796	−0.3	TRACE

+ + + High abundance; + + Low to medium abundance; NA-not applicable.

glucose, yeast extract and different inorganic nitrogen supplementation (NH_4Cl or NH_4NO_3, equal amounts of N) AZI removal was generally slow and rather similar in the presence of both inorganic N sources, with $t_{1/2}$ values of approximately 5 days (Table 1). By contrast, no removal was observed in the presence of a higher concentration of NH_4NO_3 (200 mg/L). Further adaptation of activated sludge to AZI under the same initial conditions during the next 2 months led to a faster AZI removal. The $t_{1/2}$ values were approx. two times shorter in the presence of NH_4Cl and approx. 1.4 times shorter in the presence of NH_4NO_3 as compared with cultures tested after 2 months of enrichment. NH_4Cl was therefore selected to serve as the sole N source in further experiments. As evident from Table 1, removal of AZI, added as the sole organic C source, was slightly faster than that of AZI under cometabolic conditions with glucose, yeast extract and NH_4Cl supplementation. Under both conditions, microbial cell growth was inhibited during the 10 days of incubation and observed only on the last day of the experiment (day 13). The maximum specific growth rate (μ_{max}) on AZI as the sole organic C source was 1.53 times lower than that observed in the presence of glucose as an additional organic C source.

3.2. Biodegradation of macrolide antibiotics by enriched sludge culture

The sludge culture enriched in the presence of AZI (10 mg/L) over a period of 4 months was used to study biodegradation efficiency and removal kinetics of three prominent macrolide antibiotics, AZI, ERY and CLA (Fig. 1). The enriched microbial culture exhibited the ability to degrade all three macrolide antibiotics, added as a sole organic C sources at the initial concentration of 10 mg/L. The removal curves of studied compounds as well as the abiotic control and microbial growth curves are presented in Fig. 2. No significant changes were observed in any of the abiotic controls, which confirmed that the observed removal can be attributed primarily to biological transformations. The possible impact of phototransformation [14] was excluded by performing the experiments in the dark.

The final elimination efficiency achieved after prolonged exposure of 160 h exceeded 99% for all investigated compounds. However, the corresponding removal kinetics of individual macrolides was rather different. The removal curves of AZI, ERY and CLA (Fig. 2) were fitted with the two mathematical kinetic models: the first-order kinetic model and the logistic model. The kinetic parameters for the model that provided a better fit for each of the investigated macrolide compounds are presented in Table 2.

The degradation kinetics of AZI, which can be better described by logistic ($R^2 = 0.95$) than the first-order kinetic model ($R^2 = 0.87$), showed the $t_{1/2}$ value of 3.4 days and degradation rate constant of $1.66 \pm 0.22\ day^{-1}$. Several publications have already demonstrated

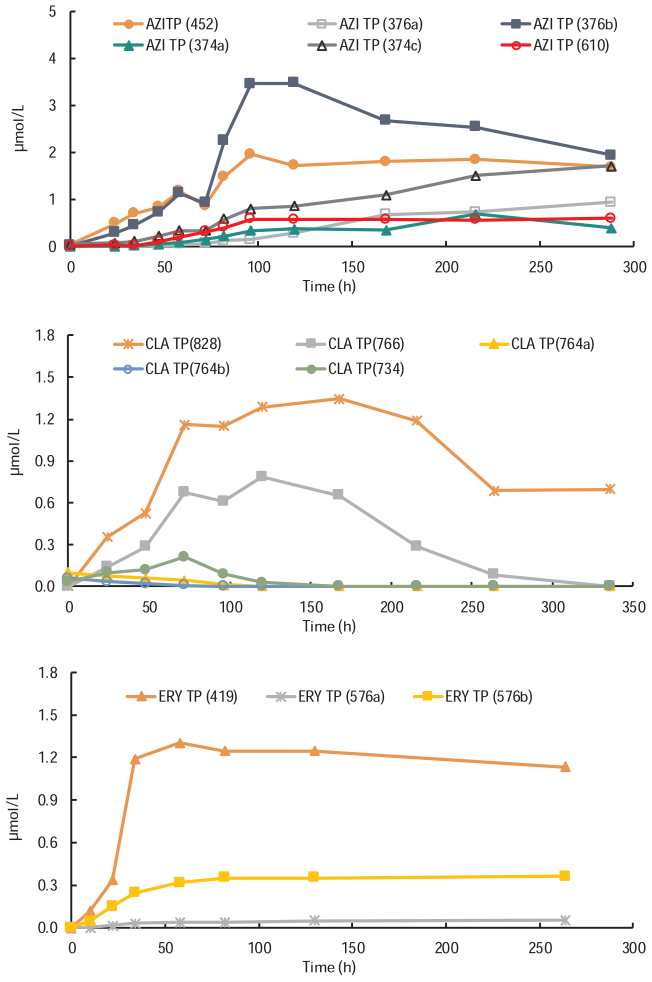

Fig. 3. Temporal changes in the concentration of the most prominent transformation products (TPs) of azithromycin (AZI), clarithromycin (CLA) and erythromycin (ERY) determined in biodegradation experiments.

that the logistic model was applicable to describe degradation of some sulfonamide antibiotics [28,29]. It is also interesting to note that AZI removal was not accompanied by concomitant cell growth (Fig. 2). Moreover, at the beginning of the experiment an instant decrease of AZI concentration (about 20%) was observed, which can be attributed to adsorption on the inoculated biomass. This is in accordance with the moderately high K_d value (about 500 L/kg) of AZI [5] However, at the end of the experiment, when practically all AZI was removed from the dissolved phase, the amount of AZI bound onto biomass was a minor fraction of the overall AZI removal mass balance (less than 1%).

By contrast, the removal of ERY and CLA followed first-order kinetics. The $t_{1/2}$ values of CLA and ERY removal were 2.1 days and 0.57 days, respectively, which indicated faster biodegradation of these two macrolide antibiotics as compared to AZI. Since the K_d values of ERY and CLA are lower than that for AZI [5], the amount of these macrolides bound to sludge can be considered negligible. It should also be stressed that the removal of the natural macrolide ERY was much faster than the removal of both semi-synthetic macrolides. Moreover, unlike for AZI, a significant and immediate cell growth was observed during ERY biodegradation, whereas during CLA degradation the cell growth started after a lag time of approximately 5 days (Fig. 2).

3.3. Biotransformation products and tentative biotransformation routes

Microbial elimination of all studied parent macrolide antibiotics was associated with the formation of a number of different transformation products (TPs). Figs. S1–S3 in the Elecronic Supplementary Material show the corresponding total ion chromatograms of the biodegradation media at the beginning of the experiment and after the progressive degradation of the selected compounds acquired in the PI and NI mode. The full list of TPs identified in the PI mode, including their retention times, elemental composition, *m/z* values, mass errors as well as their relative abundance, is presented in Table 3. The TP names listed in Table 3 were derived from the *m/z* values of their corresponding $[M+H]^+$ ions and these assignments were also used in Table S1, which lists the TPs detectable in the NI mode. It should be noted that all TPs detected in the NI mode were also detectable in the PI mode, so the Table 3 contains a full list of TPs identified in this work. The structural identification of the detected TPs was performed based on the elucidation of the accurate mass-spectrometric data and MS2 experiments, and the reporting of identification confidence followed the five-level system proposed by Schymanski et al. [30]. Results obtained in the NI mode were used for confirmation, in particular to reveal the presence of the carboxylic acid moiety in some TPs formed after opening of the macrolide ring. It should be stressed that, for the previously known TPs, the identifications were performed based on recent literature data on macrolide TPs identified in municipal wastewaters and/or during transformation studies [9,21,31–33] and, if available, using authentic reference standards. The tentative structural elucidation of the novel TPs was performed using the line of evidence approach based on expert interpretation of accurate mass spectra, including isotopic pattern analysis, MS/MS experiments and assuming some of the known mechanisms of oxidative transformation, which in most cases allowed identification of a probable structure (confidence level 2a). The known inactivation mechanisms of macrolide antibiotics such as phosphorylation, glycosylation and the hydrolysis of the macrolactone ring [22] were also considered and were an essential guidance during the structural elucidations. A similar methodological approach, making use of the common enzymatic reactions involved in the metabolism of xenobiotics, was applied to study biotransformation pathway of some biocides and pharmaceuticals [34].

3.3.1. Azithromycin

The biotransformation of AZI in our experiments resulted in the formation of a rather high number of different TPs (Table 3, Fig. 3), some of which were rather abundant and previously unreported in the literature. The confidence levels of the proposed identifications are presented in Table S2 (Electronic Supplementary Material). The total number of the detected AZI TPs included 20 different compounds whose total concentration gradually increased during the first 100 h and remained rather stable until the end of the experiment (266 h). Moreover, the semi-quantitative estimates, which were made by assuming the same molar responses of TPs and their parent compounds, indicated that the total concentration of all AZI TPs (obtained by summing up the concentrations of all identified TPs expressed in µmol/L) at the end of the experiment (266 h) represented almost 80% of the initial AZI concentration (Fig. S4). These findings represent a strong indication that some of AZI TPs identified in our experiments might play a rather important role in the overall mass balance of AZI-related compounds in WWTPs. Our preliminary study in the WWTP of the city of Zagreb [9], indeed confirmed the importance of some TPs identified in this study.

A couple of the detected compounds belonged to the group of AZI TPs with an intact macrolactone ring, which have previously been reported in the literature (e.g. [14,21,31,32]). These include TPs which were formed either by the removal of one or both sugar units (AZI TP (434); AZI TP (592)) or by some modification of desosamine sugar moiety such as *N*-demethylation (AZI TP (735)), *N*-oxidation (AZI TP (765b)) and phosphorylation (AZI TP (829)). However, it should be stressed that these TPs represented only a minor fraction of the total AZI TPs detected in the media after the removal of the parent compound.

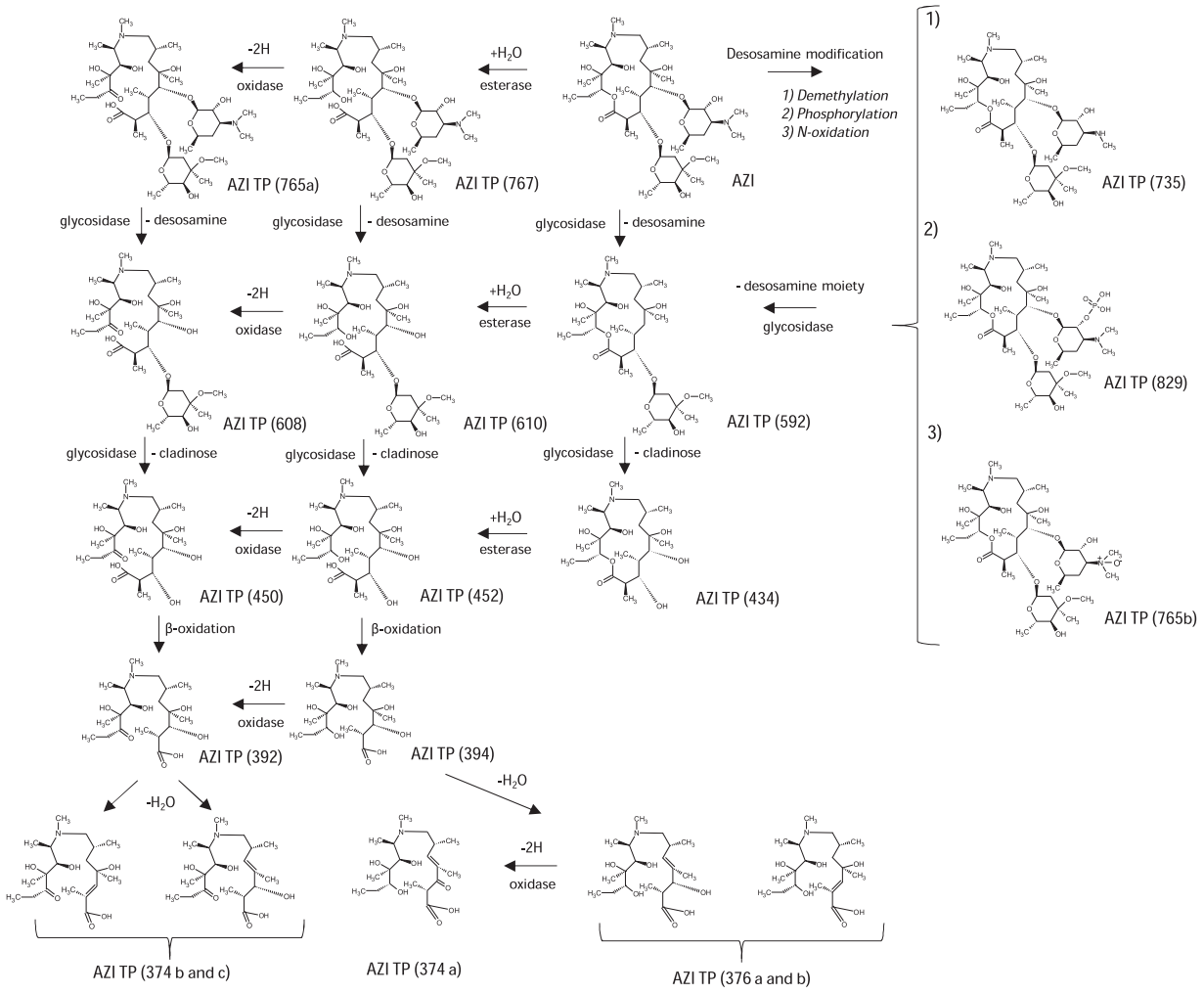

Fig. 4. The proposed tentative biotransformation pathway of azithromycin (AZI).

By contrast, the most prominent AZI TPs formed in our biotransformation experiments primarily included previously unreported TPs (e.g., AZI TP (610), AZI TP (452), AZI TP (394), (AZI TP (376a-b) and AZI TP (374a, c)). These TPs represented 80–94% of the total (summed up) concentration of all AZI TPs determined throughout the experiment. These TPs were chromatographically shifted to the left (Fig. S1, Table 3), indicating that they possessed more polar or less lipophilic character than AZI, which is in accordance with expected oxidative changes. Additionally, their even *m/z* values indicated that, unlike AZI, these compounds contained only one nitrogen atom, most probably due to the loss of desosamine. Furthermore, some of these prominent TPs were characterized by *m/z* values lower than 434, which suggested that the biotransformation must have included opening and further modifications of the macrolactone ring. Therefore, based on the obtained chromatographic and mass-spectrometric data, including accurate mass spectra and MS/MS experiments (Table 3, Fig. S5-1-10) as well as on the existing knowledge on the main inactivation mechanisms of macolide antibiotics [22], we proposed a tentative biotransformation pathway of AZI which includes several crosslinked biotransformation routes (Fig. 4).

One of the key initial steps in AZI transformation is enzymatic hydrolytic opening of the macrolactone ring, most probably mediated by the enzyme macrolide esterase Ere [22], which resulted in the formation of AZI TP (767). Its further degradation included the subsequent enzymatic cleavage of the desosamine ($-C_8H_{15}NO_2$; –157.1103 Da) and cladinose ($-C_8H_{14}O_3$; –158.0943 Da) moieties, leading to the formation of AZI TP (610) and AZI TP (452), respectively. Further transformation of AZI TP (452) to AZI TP (394) was probably achieved by β–oxidation, which resulted in a net loss of C_3H_6O (58.0419 Da) at the head of the linearized molecule. The next two biotransformation steps included two subsequent water losses, which could have occurred at two different positions, producing therefore two isobaric TPs (AZI TP (376a and b)) and AZI TP (358). All these TPs contained a free carboxylic moiety which allowed their confirmation in the NI mode (Table S1).

AZI TP (610) and AZI TP (452) could have also been formed by hydrolytic linearization of the corresponding precursors AZI TP (592) and AZI TP (434), both having the intact macrolactone ring. The latter two TPs were produced by a subsequent enzymatic removal of desosamine and cladinose moieties from AZI itself and/or from the TPs previously formed by modifications of desosamine unit (AZI TP (735); AZI TP (765b); AZI TP (829)).

An additional AZI degradation route included the production of AZI TP (765a), most probably by the oxidation of the hydroxy group of AZI TP (767) at position 13, whereas its further degradation probably followed the steps proposed for the degradation of AZI TP (767), leading to the formation of AZI TP (608), AZI TP (450), AZI TP (392) and AZI TP (374), respectively. However, the latter 4 AZI TPs could have also been formed by the oxidation of one of the OH groups of AZI TP (610), AZI TP (452), AZI TP (394) and AZI TP (376), in most of the cases at the position 13. The identity of the major TPs, AZI TP (450), AZI TP (392) and AZI TP (374), was also confirmed in the NI ionization mode, whereas the trace TPs AZI TP (765) and AZI TP (608) were not detectable due to the low sensitivity in the NI mode.

The corresponding MS/MS spectra with a more detailed description

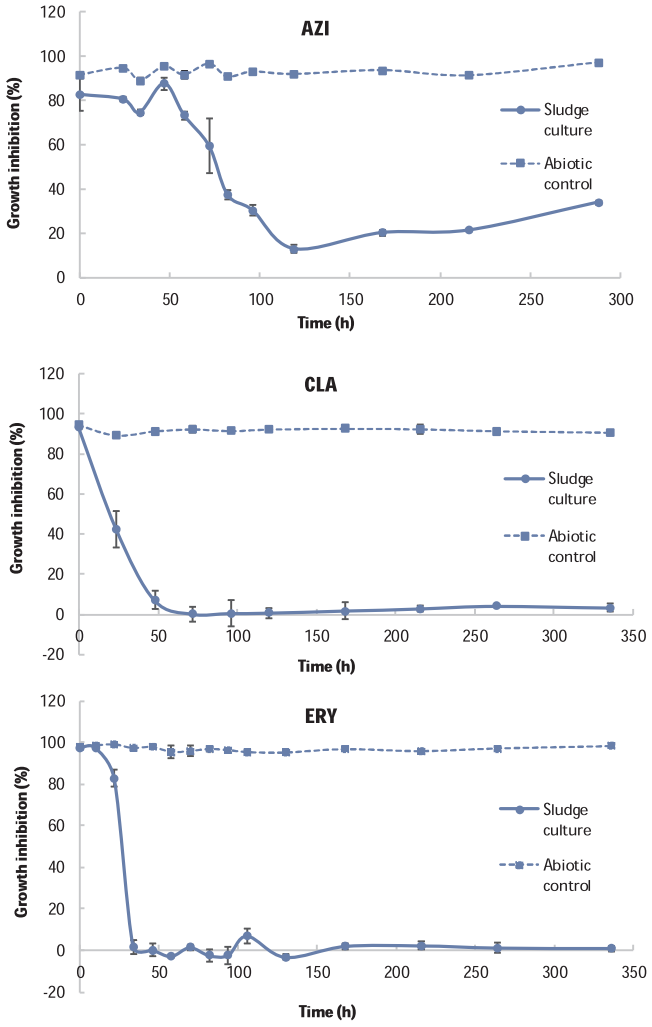

Fig. 5. Ecotoxicological evaluation of biotransformation of azithromycin (AZI), clarithromycin (CLA) and erythromycin (ERY) as reflected by corresponding changes in antibiotic activity. Test organism: *Bacillus subtilis* Marburg, ATCC 6051 culture.

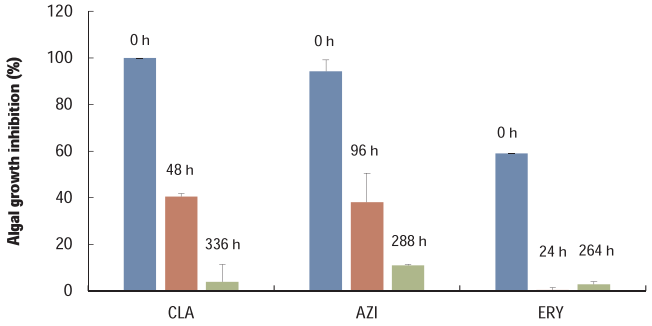

Fig. 6. Ecotoxicological evaluation of biotransformation of azithromycin (AZI), clarithromycin (CLA) and erythromycin (ERY) as reflected by corresponding changes in algal toxicity. Test organism: *Desmodesmus subspicatus* (86.81 SAG).

of their structural elucidation are given in the Electronic Supplementary Material (Figs. S5-1 to S5-10).

3.3.2. Erythromycin and clarithromycin

The number of TPs detected in ERY and CLA biotransformation experiments (Table 3, Figs. S2, S3, S6 and S7) as well as their abundance in terms of percentage of the initial parent compound concentration were much lower than for AZI. The semi-quantitative estimates of the concentrations of CLA and ERY TPs were made by assuming the same molar responses of individual TPs and their parent compounds (Fig. 3). The total concentrations of CLA and ERY TPs were in the range of 0.2–2.1 µmol/L and 0.2–1.7 µmol/L (Fig. S4), respectively. The highest levels represented 11% and 17% of the initial concentration of the corresponding parent compounds, respectively. There is a possibility that some highly polar TPs could have been eluted with the dead volume and remained undetected. Nevertheless, such a low percentage of TPs in the overall mass balance and absence of additional peaks in the LC-MS traces acquired in the PI and NI mode may suggest that a significant part of CLA and ERY could have been removed by ultimate degradation to carbon dioxide. By contrast, this was not the case during the degradation of AZI. The reason is likely the structure of the macrolide ring, which for AZI includes an additional N-atom at the 9a position. This probably brings about an enhanced persistence of AZI-derived structures.

The mass spectral evidence showed that TPs of ERY and CLA were formed by the same general mechanisms as described above for AZI. The most prominent ERY TPs, ERY TP (576) and ERY TP (419), were formed by the consecutive enzymatic cleavage of the cladinose and desosamine units. These two TPs represented over 95% of the total TP concentration throughout the experiment. The maximal concentration of ERY TP (419) coincided with the complete removal of ERY (about 30 h) and remained rather stable until the end of the experiment (266 h) (Fig. 3). Several minor ERY TPs were produced by modification of desosamine moiety, which included phosphorylation (ERY-TP (814), *N*-oxidation (ERY TP (750)) and *N*-demethylation (ERY TP 720)). Another minor TP (ERY-TP (750a)) was formed by an enzymatic hydrolysis of the macrolactone ring [22] with subsequent oxidation of one OH group. The TPs reported in the study by Kwon [16], including anhydroerythromycin and erythromycin enol ether, were not found to be products of biotransformation in our experiments.

The most prominent CLA TPs, which represented 70–100% of all detected TPs, were CLA TP (828) and CLA TP (766). These two TPs were formed by phosphorylation of desosamine and by enzymatic hydrolysis of the macrolactone ring, respectively. The highest concentrations of the main CLA TPs were found in the time-frame between 72 to 216 h, whereas the later phase of the experiment was characterized by their pronounced decrease. Other CLA TPs were present only at trace levels and included CLA TP (764a), CLA TP (764b) and CLA TP (734), which were identified as 14-OH CLA, CLA-N oxide and *N*-demethyl CLA, respectively. The presence of 14-OH CLA and *N*-demethyl CLA in municipal WWTPs have been recently reported in the literature [9,19].

3.4. Toxicity evaluation

The biotransformation of all 3 investigated macrolide antibiotics was associated with a decreasing residual antibiotic activity (Fig. 5) and algal toxicity (Fig. 6). However, some differences were observed between AZI, whose elimination was associated with the formation of rather numerous and stable TPs, and the remaining two macrolides. Namely, after 100 h of degradation the residual antibiotic activity of CLA and ERY dropped to almost zero, whereas the residual activity in AZI degradation experiment remained clearly detectable and even increased towards the end of experiment (15–30%). The increase of antibiotic activity was concomitant with the emergence of the stable TPs, such as AZI TP 374b, AZI TP 374c, AZI TP 392, AZI TP 376a and AZI TP 450 (Fig. S8 in Electronic Supplementary Materials). These compounds are structurally rather different from the parent compound; and it should be assumed that the underlying molecular mechanism of their antibiotic/bacteriostatic activity should also be different. Namely, the antimicrobial activity of the parent macrolides relies on the structural features, which include a macrocyclic lactone ring and the preserved dimethylamino group of the desosamine moiety [22]. All these features were lost in the first phase of the AZI biotransformation before the emergence of the TPs that coincided with the increase of antibiotic activity. Unfortunately, the novel TPs identified in this study were not

available as reference materials to confirm this hypothesis. It should be stressed that CLA and ERY transformation did not result in the formation of compounds analogous to AZI TPs that were assumed responsible for the residual antibiotic activity. Removal of antimicrobial activity was also reported during the oxidation of macrolide antibiotics by ozone and hydroxyl radical [11]. In contrast to our findings, the decrease of antibacterial activity in that report was highly correlated with the elimination of macrolides with no residual activity at the end of the experiment.

Algal toxicity is an important end-point to assess the ecotoxicological effects of the removal of macrolide antibiotics since algae are generally much more sensitive than bacteria [35,36]. The EC50 values for chronic toxicity of AZI and CLA to freshwater alga *Pseudokirchneriella subcapitata* were 0.019 and 0.012 mg/L [37], respectively. The corresponding value for ERY was 0.020 mg/L [35]. In our experiments, a marked decrease of toxicity coincided with the removal of parent compounds. The residual toxicity in all experiments was lower than the estimated percentage of stable degradation products (Fig S4), indicating that the formed TPs were less toxic to algae than the parent compounds. Such a situation revealed biotransformation as an ecotoxicologically favorable process. Baumann et al. [38] indicated that some of the metabolites, notably 14-OH CLA, can be as toxic as the parent macrolides; however this metabolite was only a minor CLA TP in our experiments and therefore cannot explain the residual activity. Nevertheless, significant accumulation of stable TPs in the experiments with AZI, reaching 80% of the initial parent compound concentration, can probably be linked to slightly enhanced algal toxicity in that medium. This issue, combined with the observed residual antibiotic activity, warrants further investigation.

4. Conclusions

Biotransformation is an attractive strategy to reduce the exposure to antimicrobials and proliferation of antibiotic resistance via the aquatic route. However, for macrolide antibiotics which are not easy to degrade, this goal can be achieved only by using enriched microbial cultures. Our study showed that a microbial culture enriched from the activated sludge of a municipal WWTP was capable of degrading high concentrations (10 mg/L) of three prominent macrolides, erythromycin, clarithromycin and azithromycin, which could be particularly important for the treatment of heavily polluted industrial wastewaters. It should be stressed that ERY and AZI have recently been proposed for inclusion into the EU Watch List as emerging contaminants of concern. Although the effect-driven evaluation of the biotransformation process, based on toxicity to algae and residual antibiotic activity, indicated a significant reduction of harmful effects, the formation of numerous stable metabolic products warrants further ecotoxicological assessment.

Conflict of interest

The authors of this study declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2018.01.055.

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