Analysis and occurrence of macrolide residues in stream sediments and underlying alluvial aquifer downstream from a pharmaceutical plant

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Abstract

Macrolide antibiotics azithromycin (AZI), erythromycin (ERY) and clarithromycin (CLA) have been recently included in the EU Watch List of contaminants of emerging concern in the aquatic environment. However, their comprehensive assessment in different environmental compartments, by including synthesis intermediates, by-products and transformation products, is still missing. In this work, a novel method, based on pressurized liquid extraction and liquid chromatography–tandem mass spectrometry, was developed and validated for the determination of such an extended range of macrolide residues in sediment and soil samples at low ng/g levels. The method was applied to determine distribution of 13 macrolides in surface and alluvial aquifer sediments collected in a small stream with a history of chronic exposure to wastewater discharges from AZI production. The total concentrations of the target macrolide compounds in surface sediments were up to 29 µg/g and the most prominent individual macrolides were parent AZI, its synthesis intermediate *N*-demethyl AZI and transformation products decladinosyl AZI and *N'*-demethyl AZI. Some ERY-related compounds, originating from AZI synthesis, were also frequently detected, though at lower concentration levels (up to 0.31 ng/g in total). The distribution of macrolide residues in surface sediments indicated their active longitudinal transport by resuspension and redeposition of the contaminated sediment particles. The vertical concentration profiles in stream sediments and the underlying alluvial aquifer revealed that macrolide residues reached deeper alluvial sediments (up to 5 m). Moreover, significant levels of macrolides were found in groundwater samples below the streambed, with the total concentrations reaching up to 1.7 µg/L. This study highlights the importance of comprehensive chemical characterization of the macrolide residues, which were shown to persist in surface and alluvial aquifer sediment more than ten years after their discharge into the aquatic environment.

Keywords: antibiotics, azithromycin, groundwater, erythromycin, transformation products

Capsule: Macrolide residues persist in pharmaceutically-impacted stream sediments more than ten years after the discharge and reach deeper alluvial sediments and groundwater aquifer.

1. Introduction

Pharmaceutical compounds are continuously released into the environment and, consequently, their ubiquitous occurrence in different environmental compartments has been confirmed by numerous studies in the last 20 years (aus der Beek et al., 2016). Among many classes of pharmaceuticals, a special attention has been given to antibiotics and other antimicrobial substances, largely due to the possible contribution of their residues in the environment to the development and spread of the resistant bacterial strains (Baquero et al., 2008). Antibiotic resistance is nowadays a growing problem and one of the major global challenges related to human health, food security, and development (WHO, 2018).

Macrolide antibiotics, including azithromycin (AZI), erythromycin (ERY) and clarithromycin (CLA), are used to treat different infections caused by both Gram-positive and Gram-negative bacteria, with enhanced activity against the atypical respiratory pathogens (Alvarez-Elcoro et al., 1999). They are highly ranked both regarding their consumption rate (Van Boeckel et al., 2014) and possible adverse effects to the environment (Isidori et al., 2005; Rodriguez-Mozaz et al., 2015). Several studies indicated their potential harmful effects to non-target species (Yan et al., 2019; Machado and Soares, 2019). In fact, AZI, CLA and ERY, the most prominent macrolide antibiotics, were the first antimicrobial compounds to be included in the EU Watch List of contaminants of emerging concern in the aquatic environment (European Commission, 2015).

Numerous studies in the last two decades investigated environmental occurrence and fate of macrolide antibiotics. However, most of them were focused on water matrices, such as wastewater and/or surface water (Göbel et al., 2007; Paíga et al., 2016; Rodriguez-Mozaz et al., 2015; Senta et al., 2013; Sousa et al., 2019; ter Laak et al., 2010), and groundwater (Boy-Roura et al., 2018; Jurado et al., 2019; López-

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Serna et al., 2013). Several studies pointed out that macrolide antibiotics, especially AZI, could have a rather high sorption affinity to solid matrices (Boy-Roura et al., 2018; Gibs et al., 2013; Hanamoto and Ogawa, 2019; Senta et al., 2013; Vermillion Maier and Tjeerdema, 2018), which might lead to their retardation and accumulation in soil and sediment samples. Nevertheless, their occurrence and fate in solid environmental matrices has been less studied (Fernandes et al., 2020; Liang et al., 2013; Yang et al., 2010). In addition, only few studies assessed the relationship between macrolide concentrations in aqueous and sediment phase in surface waters. In most cases, concentrations in the dissolved phase were in the low ng/L range (Azuma et al., 2017; Carmona et al., 2017; Li et al., 2019), although higher concentrations, reaching low µg/L levels, were occasionally observed (Fernandes et al., 2020). Concentrations in sediments, determined in these studies, were mostly in the low ng/g range. Moreover, the macrolide levels in aqueous and sediment environmental compartments were not always well-correlated. Recent study by Liu et al. (2019) on persistence and migration of antibiotics in streams, using a simulated hydrodynamic system, showed that photolysis was the major attenuation process for ERY and roxithromycin (ROX), followed by sorption and biodegradation.

Apart from anhydro erythromycin (ERY-H$_2$O), main metabolite of ERY, most of the studies on environmental occurrence and fate of macrolide antibiotics were focused only on parent antibiotics, while their metabolites were investigated only occasionally (Gracia-Lor et al., 2014; Senta et al., 2019), and synthesis intermediates, byproducts and transformation products were mostly neglected. However, our recent study at the wastewater treatment plant of the city of Zagreb (Croatia), emphasized their possible importance to the overall mass balance of macrolides in municipal wastewater (Senta et al., 2019). Moreover, although urban wastewater is considered to be the most important global source of input of antibiotic-related substances in the environment, pharmaceutical industry facilities should not be neglected as additional, point source of input of these emerging contaminants at specific locations (Schafhauser et al., 2018).

Our earlier non-target screening analysis of freshwater sediments chronically exposed to pharmaceutical industry effluents showed that the parent macrolides AZI and ERY were present in high concentrations, but also revealed a significant presence of AZI synthesis intermediate *N*-demethyl azithromycin (N-DM-AZI) (Terzic and Ahel, 2011). A detailed study of the pharmaceutical industry effluents using fully quantitative approach showed that concentrations of synthesis intermediates and by-products can be similar or even higher than the levels of parent AZI (Senta et al., 2017), reaching often mg/L range. These effluents were shown to have a range of negative impacts on the receiving Sava River (Bielen et al., 2017). Moreover, it was shown that such an exposure significantly increased the relative abundance of antibiotic resistant microorganisms and caused bacterial community shifts in impacted river sediments (Milaković et al., 2019).

Therefore, the aims of this work were: 1) to develop and validate a novel analytical method for determination of a broader range of macrolide compounds, including parent antibiotics, their metabolites, synthesis intermediates, by-products and transformation products, in sediment and soil; 2) to investigate the occurrence and distribution of these compounds in surface and alluvial aquifer sediment samples, as well as in groundwater at the location which was chronically exposed to discharges from the local pharmaceutical industry.

2. Experimental section

2.1. Study area and selection of the target compounds

Gorjak stream is a small tributary of the Sava River, located approximately 20 km west from the city of Zagreb. Its hydrology depends mainly on local precipitation and in dry periods a significant percentage of its total flow derived from the local industry effluents. For several decades, until 2007, the stream served as a recipient of untreated industrial wastewater effluents from the baker's yeast and

pharmaceutical production. In the last decade, dramatic changes of the stream hydrology have been observed. As a result of a climate-related lower stream base-flow and re-direction of the industrial effluents into the public sewer system, the stream evolved from a system with a permanent flow to a predominantly dry streambed.

Among other active pharmaceutical ingredients, the pharmaceutical facilities discharging wastewater into the Gorjak stream have been most notable for their large production of macrolide antibiotics, in particular AZI. AZI synthesis, shown in Fig. 1, involves ERY as a precursor, while erythromycin oxime (ERY-OX), erythromycin imino ether (ERY-IE) and N-DM-AZI are intermediates in this process. All these compounds were included in our method. However, it should be noted that ERY was determined in the form of ERY-H_2O. ERY, as a rather unstable compound, converts quickly to ERY-H_2O under metabolic and non-metabolic conditions, especially at low pH (Senta et al., 2008).

Apart from the substances directly involved in AZI synthesis, some additional AZI- and ERY-related compounds were also included in the method. Decladinosyl azithromycin (DC-AZI) and *N*'-demethyl azithromycin (N'-DM-AZI) are formed as minor by-products during AZI synthesis, however they can also derive from other processes, such as human metabolism and microbial transformation of AZI (Senta et al., 2019). Similarly, erythromycin enol ether (ERY-EE) and *N*-demethyl erythromycin (DM-ERY) are both transformation products of ERY, although DM-ERY might also originate from the human metabolism of ERY.

Another macrolide antibiotic from the EU Watch list – CLA, was also included in the new method, along with its possible transformation products – decladinosyl clarithromycin (DC-CLA) and *N*-demethyl clarithromycin (DM-CLA). To our knowledge, CLA has not been produced at the investigated location. The same applies to ROX, for which only parent compound was included in the analyses.

Structures of all 13 macrolide compounds included in the analytical method for solid environmental matrices are presented in Electronic Supplementary Material (Fig. S1).

2.2. Chemicals and materials

AZI, ERY-IE, ERY-OX and CLA were kindly supplied by Pliva (Croatia), while ROX was acquired from Sigma-Aldrich (Germany). The remaining eight target compounds, as well as deuterated azithromycin (AZI-d_3) and deuterated clarithromycin (CLA-d_3), used as internal standards, were purchased from Toronto Research Chemicals (Canada).

HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from BDH Prolabo (UK), acetone from Merck (Germany), anhydrous ammonia solution (7 N in MeOH) from Alfa Aesar (Germany), and ACS grade formic acid, as well as quartz sand (SiO_2), from Sigma-Aldrich. Ultrapure water was produced by Elix-Milli-Q system (Millipore, USA). Cellulose filters (type D28), used for pressurized liquid extraction (PLE), were produced by Dionex Corporation (USA) and regenerated cellulose filters (0.45 μm), used for the final extract filtration, by Waters (USA). HPLC column ACE C18 PFP (150 × 3 mm; 3 μm) was purchased from Advanced Chromatography Technologies (UK).

2.3. Sample collection and pre-treatment

Sampling campaign for sediments was performed in October 2018. Surface sediment samples were collected at eight locations along the Gorjak streambed. Reference location (A) was situated approximately 200 m upstream of the pharmaceutical industry facilities, while the remaining seven locations (B–H) were all situated downstream of the facilities. In addition, at four locations (B, C, D and G), the underlying alluvial aquifer sediments were also collected at four depths (vertical profiles).

Sampling details and the main characteristics of the collected samples can be found in Table 1, while the locations coordinates are listed in Table S1. It should be pointed out that during the sampling campaign, Gorjak stream was almost completely dry and only on few micro-locations, including sampling point B, shallow water layers (<10 cm) could be observed. To our knowledge, this situation was rather common in the last 10 years, and, consequently, at most locations, surface sediment had soil characteristics. Surface sediment samples were collected using stainless steel spatula, while core samples were obtained by boring with a rotary drilling rig (Comacchio GEO 205, Riese Pio X, Italy). The cores were visually inspected and, based on the material characteristics, composite samples were collected from distinct depths indicated in Table 1. The aquifer sediments were mainly composed of gravel and sand. Only the fraction <2 mm was collected. All samples were placed in the plastic bags. After being brought back to the laboratory, they were air-dried at room temperature for 4–5 days and then milled in an agate grinder to obtain fine homogeneous powder.

Groundwater samples from the aquifer below the Gorjak streambed were collected from the piezometers installed in the boreholes at the four locations where the cores of alluvial aquifer sediments were previously sampled. The groundwater sampling was performed several months after the installation of piezometers, in order to achieve full stabilization of the conditions in the aquifer after drilling. The sampling depth of groundwater was 2.3, 1.8, 0.3 and 0.4 m at locations B, C, D and G, respectively. After being brought back to the laboratory, the groundwater samples were stored at 4 °C and processed in less than 24 hours.

2.4. Extraction of sediment and groundwater samples

During the method development, both PLE and ultrasonic extraction (USE) were applied. PLE was performed on Dionex ASE 200 instrument (Dionex Corporation), while Sonis 10 ultrasonic bath (Iskra

PIO, Slovenia) was employed for USE. In the first preliminary experiment, which aimed to test efficiency of different solvents for the extraction of target compounds, only USE was used, and the following solvents were employed: MeOH, ACN, MeOH/acetone (80/20, *v/v*), 1% NH_3 in MeOH and 1% NH_3 in ACN. In the second experiment, performed in triplicate, the efficiency of two extraction techniques, PLE and USE, was directly compared, using the same mixture – 1% NH_3 in MeOH, which was found to be optimal in the previous experiment. In both model experiments, sample collected at the reference location (A) was extracted and the spiking level was 200 ng/g.

In the final procedure, which was applied in method validation and analysis of real samples, PLE was employed, using 0.2% NH_3 in MeOH solution. Cellulose filters were placed at the bottom of 11 mL stainless steel cells, which were then partially filled with quartz sand and a sample. In general, 1 g aliquots were extracted. However, concentrations of some target compounds were outside the method range in few samples and, in these cases, extraction was repeated using smaller aliquots (0.03–0.5 g). Internal standard mixture (100 ng of each compound in MeOH) was added to samples placed in the extraction cells, which were left open until methanol completely evaporated. Samples were then extracted in two cycles, applying the following PLE conditions: temperature 80 °C, pressure 1500 psi, preheating time 0 min, heating time 5 min, static time 10 min, flush volume 60% and purge time 1 min. Obtained extracts, whose volume was approximately 18–20 mL, were then evaporated to dryness under a nitrogen stream using a Turbovap evaporator (Caliper Life Sciences, USA) at 40 °C. Dry residues were re-dissolved in 0.5 mL of 100 mM ammonium formate/MeOH (1/1, *v/v*) mixture. Final extracts were filtered through regenerated cellulose filters before instrumental analysis to remove any residual particles.

Groundwater (1 L) was extracted on Oasis HLB cartridges, using the previously developed solid-phase extraction (SPE) method for water samples. Detailed information on the applied procedure can be found elsewhere (Senta et al., 2017).

2.5. Instrumental analysis

Our previously developed LC-MS/MS analytical method was used for instrumental analysis (Senta et al., 2017). Briefly, extracts were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on a Thermo Electron TSQ AM instrument (Thermo Electron Corporation, USA) equipped with an electrospray ionization interface. ACE C_{18} PFP HPLC column was employed for separation of the target compounds, using gradient elution with 0.1% formic acid in water (*v/v*) and ACN as eluents. Multiple reaction monitoring (MRM) mode in positive polarity was used for detection and quantification, applying two characteristic transitions for each compound. The quantification of analytes was performed applying internal standard approach. AZI-d_3 was used for AZI-related compounds (AZI, DC-AZI, N-DM-AZI and N'-DM-AZI), while CLA-d_3 was used for the remaining target macrolides. All operational parameters related to instrumental analysis are described elsewhere (Senta et al., 2017), while MRM transitions for the compounds analyzed in solid samples can also be found in Table S2.

2.6. Method validation

The following method parameters were assessed for validation: linearity, method detection limit (MDL), method quantification limit (MQL), trueness, repeatability (precision), extraction recovery and matrix effect. Linearity was determined from 10-point calibration curves obtained by injecting standard solutions containing analytes in the concentration range from 1 to 1000 ng/mL and internal standards at the fixed concentration of 100 ng/mL. Method detection and quantification limits were estimated from the instrumental detection limits reported in our previous work (Senta et al., 2017), taking into account typical sample amount and concentration factor for solid samples. All other validation parameters were assessed separately for two solid matrices – sediment and soil. They were determined in the model

experiments, performed in quadruplicate, at the spiking level of 100 ng/g. Although this study was focused exclusively on sediments, method validation for soil was also done to demonstrate the method applicability for surface sediment samples collected along the Gorjak streambed, which mostly had soil characteristics (as pointed out in subsection 2.3.). Sediment sample used for method validation was collected at the Sava River, at location approximately 2.5 km upstream the pharmaceutical industry facilities. Soil sample, with the similar texture as sediment, was collected at the reference location of the Gorjak stream. Concentrations of all target compounds in these samples were either below MQL or low enough to allow reliable method validation.

Trueness and repeatability were determined in the model experiments with sample spiked with both target compounds and IS before PLE. Trueness was then calculated from the following equation:

Trueness (%) = $(c_2 - c_1)/c_0 \times 100$

where c_0, c_1 and c_2 represent the nominal spiked concentration, average concentration measured in the original (non-spiked) sample and average concentration measured in the spiked sample, respectively. Repeatability was calculated as the relative standard deviation (RSD) of the analysis of spiked samples.

Extraction recovery was determined in the model experiments with sample spiked with target compounds either before or after PLE, taking into account analytes already present in the original (non-spiked) sample. The extraction recovery was then calculated using the following equation:

Extraction recovery (%) = $(A_{be} - A_{orig}) / (A_{ae} - A_{orig}) \times 100$

where A_{be} is the average response of analyte spiked to the sample before PLE, A_{ae} is the average response of analyte spiked to the extract after PLE and A_{orig} is the average response of analyte in the original sample.

Matrix effect was assessed by comparing the average response of analytes spiked into the final extracts (A_{fe}) with their average response in matrix-free standard solution of the same concentration (A_{std}), taking into account responses of target compounds already present in the original sample (A_{orig}). Matrix effect was then calculated using the following equation:

Matrix effect (%) = ($A_{fe} - A_{orig} - A_{std}$) / $A_{std} \times 100$

3. Results and discussion

3.1. Method development

The method development was primarily focused on optimization of the extraction procedure for a wide range of macrolide compounds. In the first preliminary experiment, efficiency of several solvents for the extraction of target compounds was tested using USE. Comparison of the extraction recoveries is presented in Fig. S2. With most of the tested solvents, recoveries were generally lower than 50%, especially with pure organic solvents (ACN, MeOH) or their mixture (MeOH/acetone). Although some previous literature reports showed acceptable recoveries from sediment samples for macrolides such as ERY, CLA and ROX (Li et al., 2012; Löffler and Ternes, 2003), our experiment clearly indicated that addition of ammonia as a basic modifier was essential for extraction improvement, especially for AZI-related compounds. Moreover, 1% NH_3 in MeOH solution (recoveries of 68% to 107%, except for ERY-EE) proved to be much more efficient than the corresponding 1% NH_3 in ACN solution (15–74%). Therefore, this mixture was selected for the further method development. It should be noted that similar solvent was already used for the extraction of ROX and ERY from soil, however in that case aqueous (instead of anhydrous) ammonia in MeOH was applied (Schlüsener et al., 2003).

Using 1% NH$_3$ in MeOH solution as an optimized extraction solvent, we compared the efficiency of two extraction techniques – PLE and USE. Results, presented in Fig. 2, show that recoveries of AZI-related compounds were mostly similar with both techniques. However, PLE recoveries of all remaining analytes (ERY- and CLA-related compounds, as well as ROX) were notably higher than the corresponding USE recoveries (in most cases the difference was 20–30%). Therefore, PLE was selected as the preferred extraction method, although it should be stressed that recoveries of two demethylated compounds (DM-ERY and DM-CLA) in this preliminary experiment were outside of the generally acceptable range (132% and 159%, respectively).

During PLE method development, problems with the ASE extractor were occasionally observed when 1% NH$_3$ in MeOH was used, presumably due to the formation of ammonia bubbles. To alleviate this problem, we lowered the percentage of ammonia in the extraction solvent to 0.2%. This modification completely resolved technical difficulties during the ASE extraction, while, at the same time, keeping the high extraction efficiency for all analytes (Table 2). This extraction solvent was applied in all further analyses, including method validation and analysis of real samples.

3.2. Method validation

Validation data for both matrices (sediment and soil) are presented in Table 2. In general, the developed methodology allows accurate and reliable determination of selected compounds, with MQLs in the low ng/g range (≤3.2 ng/g). The linearity of the calibration curves in the range from 1–1000 ng/mL was very good for all target compounds, with correlation coefficients (r^2) higher than 0.99. Depending on the sample aliquot used for LC-MS/MS analysis (0.03–1.0 g), the method's quantification range was between MQL and 15 µg/g.

Extraction recovery in sediment was in the 80–100% range, except for ERY-EE (54%). High repeatability (RSD ≤8%) and rather low matrix effect (≤7%) were also obtained for all compounds. Consequently, trueness was acceptable (83–111%), except for ERY-EE (55%), which losses during extraction could not be adequately compensated by non-ideal internal standard. Unfortunately, isotopically labelled analogues for synthesis intermediates, by-products and other transformation products are generally not commercially available and could not be included in this method.

Slightly lower extraction recovery was obtained for all compounds in soil (recoveries were generally 5–15% lower than in sediment). Nevertheless, these negligible losses during extraction were generally well-compensated by the internal standards, so trueness was again acceptable for most compounds (81–112%). The only exceptions were ERY-EE (59%) and DM-ERY (129%), which is also most probably associated with the use of non-ideal internal standard for these ERY-related substances. In addition, high repeatability (RSD ≤15%) and low matrix effect (≤12%) was obtained for all investigated compounds.

3.3. Occurrence and distribution of the target compounds in stream sediments

A complete overview of the determination of macrolide residues in stream sediments and underlying aquifer from this study can be found in Electronic Supplementary Materials (Table S3), while here we focus on the key characteristics of their distribution in the investigated system. The longitudinal distribution of the target compounds in the surface sediments of the Gorjak stream, presented in Fig. 3, shows a wide variability of the total concentrations, with a general pattern clearly indicating the location of the main input. After the wastewater discharge point (B), the total concentrations sharply decreased with the distance from the source, but remained elevated (>1 µg/g) along the whole sampling profile (about 4 km), except for the location G. As expected, the lowest total concentration was determined at the reference location A (87 ng/g), situated 200 m upstream of the pharmaceutical industry facilities,

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whereas the highest total concentration (29 µg/g) was found at the first location downstream of the pharmaceutical industry facilities (location B). It should be stressed that since 2007 the input of macrolide-containing wastewater effluents into the Gorjak stream was completely stopped by redirecting the industrial effluent to the public sewer system. The only additional input in the later period could have been associated with the aerial transport of particles (which probably explains relatively high background concentration at the upstream location A) or with a surface runoff from the production zone. Any of these two additional inputs cannot explain very high macrolide levels found on downstream locations (2.8 to 29 µg/g), leading to a conclusion that the determined levels of macrolides reflect a decade-old contamination that has persisted in the stream sediment.

The decrease of the total macrolide concentrations in surface sediments of the Gorjak stream at downstream locations was not fully regular and showed some indicative exceptions. For example, the concentration at the location G was much lower than expected, which indicated that this surface sediment was of more recent origin, lacking significant contribution of heavily contaminated particles of wastewater origin. On the other hand, the surface sediment at the location H, situated further downstream, contained rather high levels of macrolides (17 µg/g) that were comparable to the most polluted locations B and C. This strongly suggests that the longitudinal distribution of the target macrolides was a result of their gradual migration by repeated resuspension and redeposition of contaminated sediment particles. This additionally revealed that the major macrolides present in the investigated system were strongly associated with sediment particles, with a rather limited exchange with water phase, and that their transformation in sediments must have been quite slow.

With respect to their composition, 10 out of 13 target macrolides were found in surface sediments in measurable concentrations, with a strong predominance of AZI-related compounds over all other macrolides. The composition of AZI-related macrolides was strongly dominated by three compounds, including the parent AZI, DC-AZI and N-DM-AZI, whose contribution to the total macrolide load

exceeded 96%. N-DM-AZI is an important intermediate in AZI synthesis (Fig. 1), while DC-AZI is its major transformation product, which can be formed by both biotic and abiotic processes (Jaén-Gil et al., 2018; Tong et al., 2011). The contribution of N'-DM-AZI, a minor byproduct of AZI synthesis and its possible biotransformation product (Senta et al., 2019; Terzic et al., 2018), to the total concentration was less than 4%.

All ERY-related compounds, except ERY-IE, were also detected in most of the surface sediments at downstream locations. Their concentrations generally decreased in the following order: ERY-H_2O ≥ ERY-OX > ERY-EE > DM-ERY, reaching maximum of 185 ng/g, 99 ng/g, 15 ng/g and 14 ng/g, respectively. It should be noted that the concentrations of ERY-related compounds were in most cases much lower than the concentrations of AZI-related compounds. Regarding CLA-related compounds, only traces of DC-CLA (<1 ng/g), were determined in two samples, while ROX could not be detected in any of the analyzed samples.

The prevalence of AZI-related compounds can be explained with their comparatively larger inputs, but also with their higher resistance to biodegradation as compared with ERY-related substances (Terzic et al., 2018). Furthermore, AZI-like macrolides contain an additional basic nitrogen atom in the lactone ring, which significantly enhances their sorption affinity, due to the coulombic attraction to negatively charged surface sites of the sediment particles (Hanamoto et al., 2018). This results in stronger sorption and, thus, their longer retention in sediment. In fact, high soil-water distribution coefficient (K_d) and organic carbon-water partition coefficients (K_{oc}) were recently reported for parent AZI, as well as for N-DM-AZI and N'-DM-AZI (Vermillion Maier and Tjeerdema, 2018). The authors pointed out that these compounds have similar logK_{oc} (3.8–4.7) as some of the persistent organic pollutants included in the Stockholm Convention. Gibs et al. also reported very high organic carbon normalized pseudo partition coefficient (P-PC$_{OC}$) for AZI (39240 L/kg) (Gibs et al., 2013), while pseudo partitioning coefficient (P-PC) for ERY-H_2O was found to be much lower (211) (Kim and Carlson, 2007). Indeed, recent literature data

for AZI suggest that cation exchange might be the dominant sorption mechanism (Hanamoto and Ogawa, 2019). In another study, P-PC of ERY-H_2O was found to be significantly positively corelated with water pH (Liang et al., 2013). In fact, the authors suggested that sediment total organic carbon (TOC) and water column pH might be the most important factors controlling the dynamic distribution of ERY-H_2O between those two matrices.

As pointed out above, DC-AZI and N'-DM-AZI are not directly involved in AZI synthesis but were present in significant concentrations. It is unlikely that these compounds originated from the human metabolism after therapeutic consumption because the input of domestic wastewater into the Gorjak stream is negligible. Although they might be formed as minor by-products during AZI synthesis, their large relative contribution to the total macrolide loads indicated that they must have been predominantly formed *in situ*, most probably by biotransformation. This is in agreement with the study by Vermillion Maier and Tjeerdema (2018), which showed that DC-AZI was one of the major transformation products of AZI in biotransformation microcosms simulating flooded and non-flooded watershed. This hypothesis is additionally supported by a rather high correlation (r^2 = 0.96) between AZI and DC-AZI concentrations (Fig. S4). High correlation (r^2 = 0.90) was also obtained for AZI and N'-DM-AZI concentrations, indicating that microbial demethylation of AZI at the desosamine group was probably the major source of N'-DM-AZI in the Gorjak stream sediments as well. On the contrary, correlation between AZI and all compounds related to its synthesis (ERY-H_2O, ERY-OX and N-DM-AZI) was much lower ($r^2 \le 0.22$).

Since this is the first study on the occurrence of synthesis intermediates, by-products, and transformation products of macrolide antibiotics in sediments, comparison with literature data could be done mostly for the parent antibiotics. Their concentrations in freshwater sediments reported in the literature are generally lower than concentrations of parent AZI in the present study and rarely exceeded ng/g levels (Carmona et al., 2017; Feitosa-Felizzola and Chiron, 2009; Fernandes et al., 2020; Gibs et al., 2013; Li et al., 2018; Xu et al., 2014; Yang et al., 2010). Elevated concentrations of AZI were

determined in the Sava River, reflecting the impact of wastewaters from azithromycin manufacture (Milaković et al., 2019). Furthermore, comparatively high concentrations of ROX (up to 5.6 µg/g), were determined in the sediments of the Dagu River (China) (Hu et al., 2012) and the authors suggested that the major source was agriculture.

3.4. Distribution of macrolides in the aquifer sediments and groundwater

In order to assess the infiltration of individual macrolide compounds from the contaminated stream sediments into the deeper layers, we additionally investigated their distribution along the vertical profiles of underlying alluvial sediments and in groundwater. The vertical profiles of major AZI-related compounds and ERY-related compounds in alluvial sediments are presented in Fig. 4 and Fig. S3, respectively. As can be seen, the most prominent macrolides, in terms of concentration levels and detection frequency, were identical to those found in the surface sediment (AZI, DC-AZI and N-DM-AZI). However, their composition was somewhat different, with the highest molar contribution of N-DM-AZI (38%), followed by DC-AZI (35%) and AZI (27%). N'-DM-AZI was determined in all alluvial sediment samples as well, however its concentrations were notably lower and never exceeded 40 ng/g. The contribution of all other macrolide residues was lower than 1%. ERY-H_2O was found in most of the samples in low ng/g range (up to 12 ng/g), while for all other ERY-related compounds (ERY-EE, ERY-OX and DM-ERY) only traces of (<5 ng/g) were occasionally detected.

It should be noted that the concentrations of AZI-related compounds in aquifer sediments varied over three orders of magnitude (from low ng/g to low µg/g levels), but the vertical profiles of the individual compounds in the same core were very similar (Fig. 4). However, the vertical distribution profiles at different locations were rather different. At the first two downstream locations (B and C), a sharp decrease between the surface sediments and underlying alluvial sediments was observed. This indicated

a relatively inefficient infiltration into the deeper layers. A completely different vertical pattern for AZI-related compounds was obtained at the locations D and G, with the concentrations reaching maximum levels approximately 1–2 m below the surface, followed by a gradual decrease towards deeper layers. It is especially interesting to observe this pattern at location G, where the levels of all prominent macrolides (AZI, DC-AZI and N-DM-AZI) were 1–2 orders of magnitude higher than in the corresponding surface sample. In addition, a significantly enhanced level of ERY-H$_2$O was also observed in the same layer (Fig. S3). Moreover, the concentration of N-DM-AZI 1.1–2 m below the surface at location D was higher than in all remaining samples, surpassing substantially the concentration of this substance even in the most contaminated surface sediment sample (location C). Locations D and G are local depressions in the Gorjak streambed, thus acting as points of the enhanced sediment deposition. Therefore, these results indicate that the sediment layers at 1–2 m must have been deposited during the period of maximal discharges of macrolide-containing wastewater, i.e. between 1990 and 2007. The aquifer sediments below 1 m are often a part of the saturated zone of the aquifer, which enhances the possibility of the transfer of macrolide compounds to the groundwater flow. Such patches of highly contaminated aquifer sediments should be considered a significant threat to the adjacent groundwater water protection zone used as drinking water supply for 120,000 people.

The concentrations of target compounds in groundwater collected from the piezometers at four locations (B, C, F and G) are shown in Table 3. As expected, the composition and the relative abundances of individual macrolide compounds are in accordance with their distribution in sediment samples, with AZI, DC-AZI and N-DM-AZI being the most prominent residues and N'-DM-AZI, ERY-H$_2$O and ERY-OX as the minor constituents. However, two additional AZI-related compounds – phosphorylated azithromycin (AZI-PO$_4$) and azithromycin *N*-oxide (AZI-OX), which could not be determined in sediment samples due to their low extraction recovery, were found in the majority of the groundwater samples. These compounds are not expected to derive from the AZI synthesis and are highly indicative of AZI *in situ*

transformations (Senta et al., 2019; Terzic et al., 2018; Terzic and Ahel, 2011). The total concentration of target compounds was the highest at location G (1.7 µg/L), followed by locations D and B (0.29 and 0.12 µg/L, respectively), while at location C only traces of all target analytes were detected in the low ng/L range. These results are generally in accordance with the described vertical profiles of macrolides in aquifer sediments. The highest aqueous concentrations were determined for those locations where the macrolide contamination peak was found in deeper sediment layers (1–2 m), which belonged to the saturated zone of the aquifer.

Regarding the relative contribution of individual substances, some differences were noted between the sediments and groundwater samples. The highest concentrations in groundwater were observed for DC-AZI (up to 1143 ng/L) and N-DM-AZI (up to 490 ng/L), largely exceeding the concentration of the parent AZI (0.16 to 17 ng/L). The key factor leading to enhanced concentrations of DC-AZI in groundwater, as compared to parent AZI, is probably its comparatively lower partition coefficient. The experimentally determined K_{ow} value of DC-AZI is not available, but its relatively lower value in comparison to other macrolides can be inferred from its retention time on the C_{18} reverse-phase chromatographic column (Table S2). However, it is interesting to note that concentrations of N-DM-AZI also largely exceeded the concentrations of the AZI at the locations D and G, although these compounds have rather similar structures. Nevertheless, Vermillion Maier and Tjeerdema reported that the partition coefficient of N-DM-AZI was about 2.5 times larger than that of AZI (Vermillion Maier and Tjeerdema, 2018). One of the possible additional explanations would be difference in the biotransformation rates of the sediment-bound AZI and N-DM-AZI, but at this stage this assumption is only speculative and would require further research.

The comparison of our data with other literature reports is possible only for parent macrolides, due to the lack of data on transformation products. Concentrations of parent AZI in the present study were similar as in groundwater from the Baix Fluvià alluvial aquifer (Spain) (Boy-Roura et al., 2018) and

urbanized areas of Minnesota (United States) (Erickson et al., 2014), but much lower than in groundwater of the urban aquifers in Barcelona (Spain), where concentrations of AZI reached up to 1.6 µg/L (López-Serna et al., 2013). Concentrations of ERY in the same area were typically in the low ng/L range. A large study performed in the central China also showed that ERY was present in groundwater in trace concentration, despite its relatively high concentrations in surface waters (Yao et al., 2017).

It should be pointed out that the assessment of the mobility of contaminants in groundwater aquifer is a very complex issue, especially for compounds like AZI, having high octanol-water partition coefficients (K_{ow}) and the additional affinity to sediment particles based on ionic interactions (Boy-Roura et al., 2018). For AZI-related compounds it could be, therefore, expected that enhanced sorption might lead to their long retardation in sediment layers and, consequently, highly delayed emergence in the distant parts of the aquifer.

Conclusions

The developed method allows sensitive and reliable determination of the most prominent parent macrolide antibiotics, along with their metabolites, synthesis intermediates, by-products, and transformation products in solid environmental matrices (sediment and soil). The application of the method at the location chronically exposed to pharmaceutical industry effluents demonstrated importance of the comprehensive chemical characterization of the macrolide residues when assessing their fate in stream sediments and underlying aquifer. Moreover, the study clearly revealed highly recalcitrant behavior of azithromycin-related compounds in stream sediments. Due to their high sorption affinity, these persistent compounds can be retarded for a long time in surface and alluvial aquifer sediments and reach groundwater years after their discharge into the surface water bodies. Further research is needed to study the dynamics of this complex processes.

Acknowledgments

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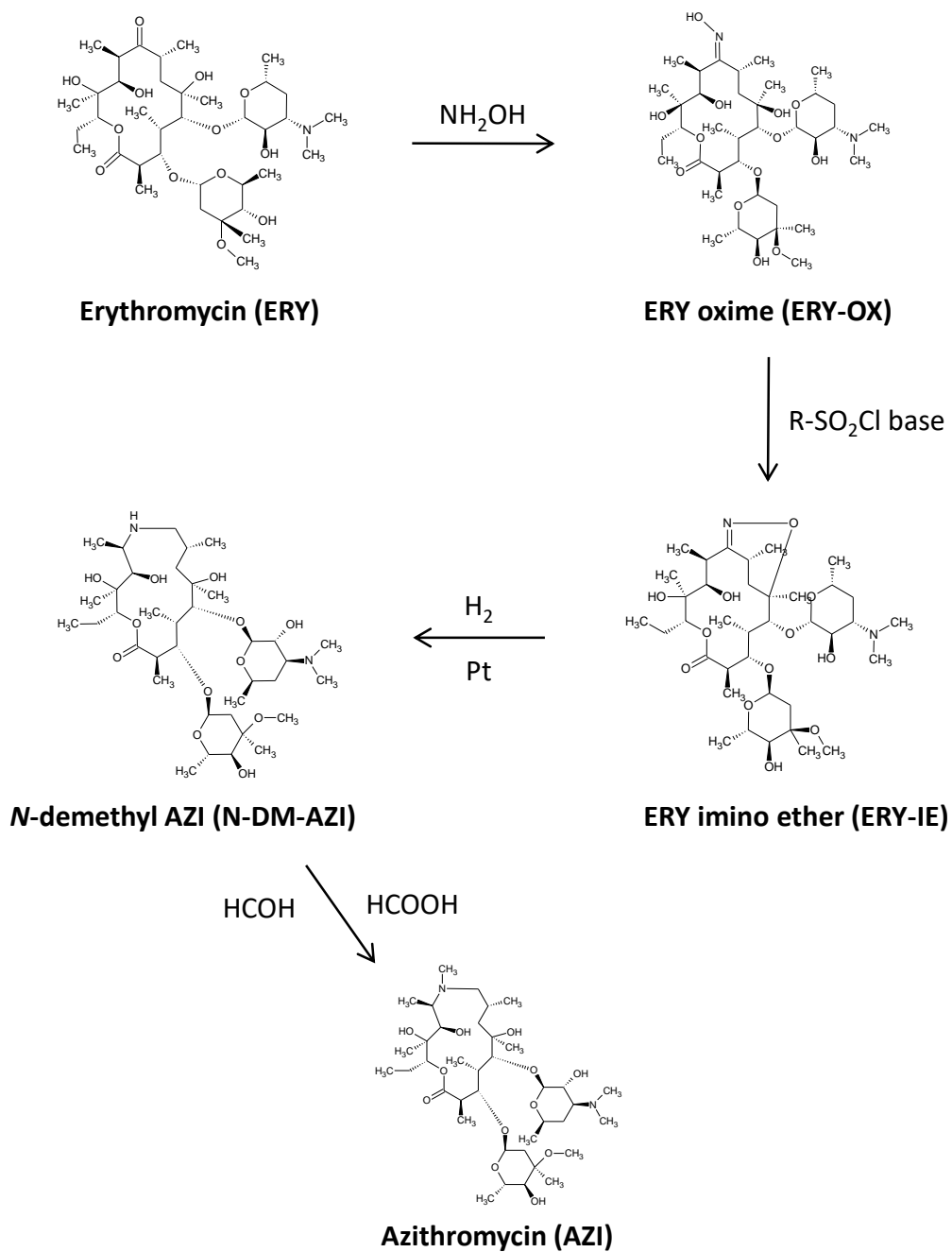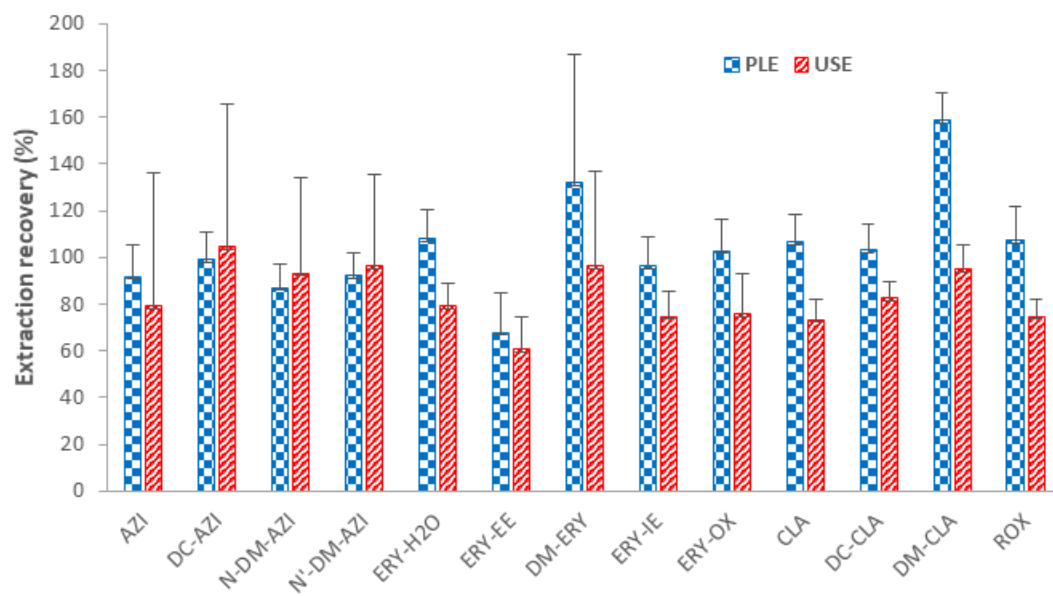**Fig. 1.** Scheme of azithromycin synthesis.

Fig. 2. Extraction recovery of the target compounds with pressurized liquid extraction (PLE) and ultrasonic extraction (USE).
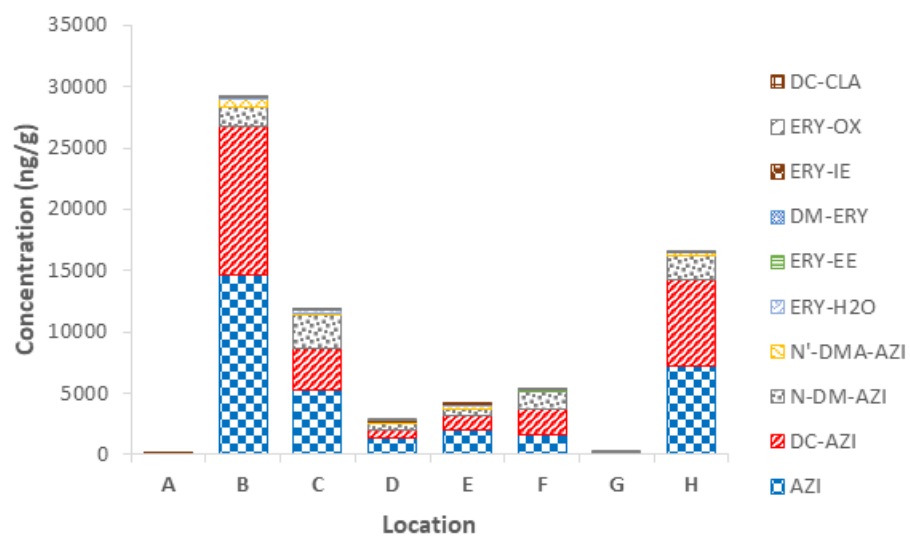
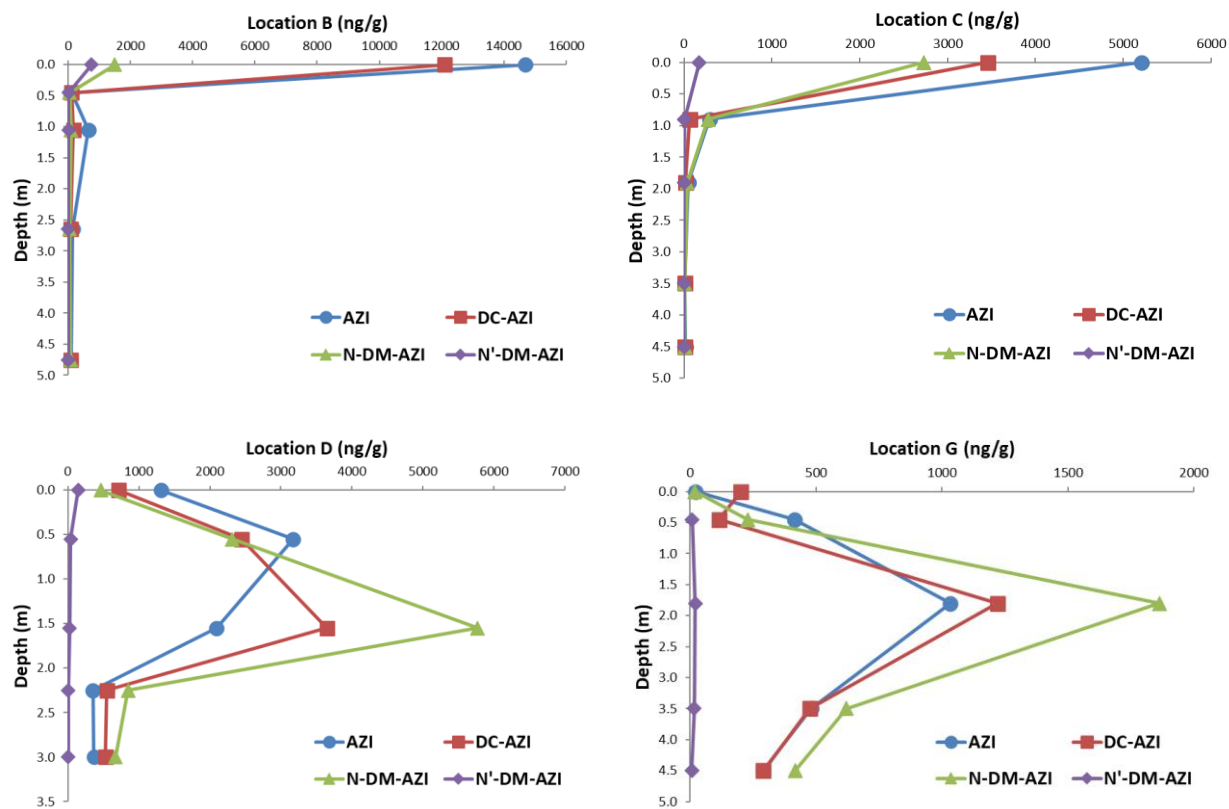**Fig. 3.** Longitudinal distribution of macrolide residues in surface sediments from the Gorjak stream.

Fig. 4. Vertical profiles of the major macrolide residues in surface and alluvial aquifer sediments from the Gorjak stream.

Table 1. Description of the Gorjak stream study area and collected sediment samples.

Location	Distance from plant	Depth (m)	Note	C (%)	N (%)	S (%)
A	200 m upstream	Surface (0–0.15)	Dry streambed	5.67	0.410	0.072
B	20 m	Surface (0–0.15)	Water layer (20 cm) Groundwater level at 2.26 m	6.79	0.262	0.241
		0.3–0.6		4.72	0.010	0.014
		0.6–1.5		3.12	0.024	0.017
		1.8–3.5		4.95	0.013	0.019
		4.0–5.5		4.77	0.009	0.009
C	600 m	Surface (0–0.15)	Dry streambed Groundwater level at 1.82 m	9.53	0.790	0.187
		0.5–1.3		4.09	0.045	0.141
		1.3–2.5		5.59	0.024	0.041
		3–4		4.72	0.010	0.022
		4–5		4.86	0.007	0.028
D	1200 m	Surface (0–0.15)	Dry streambed Groundwater level at 0.3 m	12.05	1.002	0.262
		0.1–1		5.98	0.126	0.048
		1.1–2		5.10	0.020	0.040
		2.0–2.5		4.20	0.013	0.025
		2.5–3.5		3.86	0.013	0.019
E	1700 m	Surface (0–0.15)	Dry streambed	11.80	0.996	0.273
F	2700 m	Surface (0–0.15)	Dry streambed	5.04	0.174	0.148
G	3500 m	Surface (0–0.15)	Dry streambed Groundwater level at 0.3 m	5.19	0.309	0.079
		0.3–0.6		3.38	0.027	0.025
		1.6–2.0		4.63	0.017	0.024
		3–4		3.91	0.001	0.030
		4–5		3.83	0.017	0.024
H	3900 m	Surface (0–0.15)	Dry streambed	4.60	0.271	0.097

Table 2. Method validation parameters for determination of macrolide compounds in solid environmental matrices (n=4; spiking level: 100 ng/g).

Compound	AZI	DC-AZI	N-DM-AZI	N'-DM-AZI	ERY-H$_2$O	ERY-EE	DM-ERY	ERY-IE	ERY-OX	CLA	DC-CLA	DM-CLA	ROX
Linearity (r^2)	0.9987	0.9990	0.9993	0.9967	0.9992	0.9986	0.9992	0.9924	0.9983	0.9986	0.9986	0.9993	0.9983
MDL (ng/g)[*]	0.84	1.1	1.0	0.74	0.86	0.13	0.13	0.76	0.34	0.19	0.15	0.75	0.27
MQL (ng/g)[*]	2.6	3.2	3.0	2.2	2.6	0.39	0.39	2.3	1.0	0.58	0.44	2.3	0.81
Sediment													
Extraction recovery (%)	82	91	81	82	94	54	99	92	88	89	99	91	87
Trueness (%)	97	111	94	103	105	55	93	98	83	99	107	99	97
Repeatability (%)	3	5	4	4	5	8	3	3	7	4	4	4	5
Matrix effect (%)	7	0	3	3	4	1	6	1	6	3	3	-6	5
Soil													
Extraction recovery (%)	67	85	66	69	85	50	77	76	77	80	88	80	84
Trueness (%)	99	103	81	87	110	59	129	104	100	96	112	105	104
Repeatability (%)	7	9	10	9	7	10	9	15	8	9	6	10	5
Matrix effect (%)	-12	-5	-7	-7	6	1	6	6	7	8	6	-5	7

MDL – method detection limits; MQL – method quantification limits; AZI – azithromycin; DC-AZI – decladinosyl azithromycin; N-DM-AZI – *N*-demethyl azithromycin; N'-DM-AZI – *N*'-demethyl azithromycin; ERY-H$_2$O – anhydro erythromycin; ERY-EE – erythromycin enol ether; DM-ERY – *N*-demethyl erythromycin; ERY-IE – erythromycin imino ether; ERY-OX – erythromycin oxime, CLA – clarithromycin; DC-CLA – decladinosyl clarithromycin; DM-CLA – *N*-demethyl clarithromycin; ROX – roxithromycin
[*]Based on 1.0 g aliquots

Table 3. Occurrence of macrolide residues in groundwater below the Gorjak creek bed.

Location	Concentration (ng/L)														
	AZI	DC-AZI	N-DM-AZI	N'-DM-AZI	AZI-PO$_4$	AZI-OX	ERY-H$_2$O	ERY-EE	DM-ERY	ERY-IE	ERY-OX	CLA	DC-CLA	DM-CLA	ROX
B	14	57	17	0.47	29	0.65	0.79	<0.03	<0.03	<0.05	2.4	<0.75	<0.28	<2.1	<0.06
C	0.16[*]	0.79	0.87[*]	<0.20	0.60	<0.18	<0.12	<0.03	<0.03	<0.05	<0.06	<0.75	<0.28	<2.1	<0.06
D	8.4	152	131	0.63	0.78	0.50	0.29	<0.03	<0.03	<0.05	<0.06	<0.75	<0.28	<2.1	<0.06
G	17	1143	490	<0.20	1.1	0.65	18	<0.03	<0.03	<0.05	<0.06	<0.75	<0.28	<2.1	<0.06

AZI – azithromycin; DC-AZI – decladinosyl azithromycin; N-DM-AZI – N-demethyl azithromycin; N'-DM-AZI – N'-demethyl azithromycin; AZI-PO$_4$ – phosphorylated azithromycin; AZI-OX – azithromycin N-oxide; ERY-H$_2$O – anhydro erythromycin; ERY-EE – erythromycin enol ether; DM-ERY – N-demethyl erythromycin; ERY-IE – erythromycin imino ether; ERY-OX – erythromycin oxime, CLA – clarithromycin; DC-CLA – decladinosyl clarithromycin; DM-CLA – N-demethyl clarithromycin; ROX – roxithromycin

[*] Values below method quantification limit, but above method detection limit