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Antibiotic-manufacturing sites are hot-spots for the release and spread of antibiotic resistance genes and mobile genetic elements in receiving aquatic environments

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ABSTRACT

High antibiotic releases from manufacturing facilities have been identified as a risk factor for antibiotic resistance development in bacterial pathogens. However, the role of antibiotic pollution in selection and transferability of antibiotic resistance genes (ARGs) is still limited. In this study, we analyzed effluents from azithromycin-synthesis and veterinary-drug formulation facilities as well as sediments from receiving river and creek taken at the effluent discharge sites, upstream and downstream of discharge. Culturing showed that the effluent discharge significantly increased the proportion of antibiotic resistant bacteria in exposed sediments compared to the upstream ones. Quantitative real-time PCR revealed that effluents from both industries contained high and similar relative abundances of resistance genes [*sul1*, *sul2*, *qacE/qacEΔ1*, *tet*(A)], class 1 integrons (*intI1*) and IncP-1 plasmids (*korB*). Consequently, these genes significantly increased in relative abundances in receiving sediments, with more pronounced effects being observed for river than for creek sediments due to lower background levels of the investigated genes in the river. In addition, effluent discharge considerably increased transfer frequencies of captured ARGs from exposed sediments into *Escherichia coli* CV601 recipient as shown by biparental mating experiments. Most plasmids exogenously captured from effluent and polluted sediments belonged to the broad host range IncP-1ε plasmid group, conferred multiple antibiotic resistance and harbored class 1 integrons. Discharge of pharmaceutical waste from antibiotic manufacturing sites thus poses a risk for development and dissemination of multi-resistant bacteria, including pathogens.

1. Introduction

Antibiotic resistance (AR) is rising to dangerously high levels in all parts of the world, threatening not only our ability to treat common infectious diseases, but also progress in many fields of medicine (Nicolau, 2011; O'Neill, 2016). While the overuse and misuse of antibiotics in humans and animals is undoubtedly a primary driving force for this serious problem, the environmental dimension of AR has also been recognized to play an important role in the emergence and spread of AR (Berendonk et al., 2015; Bengtsson-Palme et al., 2018; Larsson et al., 2018; Smalla et al., 2018). Antibiotic resistant bacteria (ARB) were present in the environment long time before the clinical and agricultural use of antibiotics started (D'Costa et al., 2011). These ARB and their antibiotic resistance genes (ARGs) were reported to increase in abundance whenever exposed to a sufficiently high antibiotic selection pressure (Heuer et al., 2009, 2012; Kristiansson et al., 2011; Larsson, 2014; González Plaza et al., 2018). An increasing abundance of ARGs may increase the potential for their spread to pathogenic bacteria by horizontal gene transfer (HGT) (Ghosh and LaPara, 2007). Plasmids and other mobile genetic elements (MGEs) such as integrons are regarded as key contributors in the dissemination of ARGs and promoters of multi-drug resistance (Heuer et al., 2009; Heuer and Smalla, 2012; Jechalke et al., 2013a; Flach et al., 2015; Blau et al., 2018). These MGEs are assumed to promote bacterial adaptation to rapidly changing environments (Heuer and Smalla, 2012). Plasmids belonging to the incompatibility groups IncP-1, IncN, IncQ, and IncW are of particular

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importance when studying ARGs as they have a broad host range (BHR) and may be exchanged among phylogenetically distant bacteria (Pukall et al., 1996; Suzuki et al., 2000; Shintani et al., 2010, 2014; Klümper et al., 2015). Specifically, IncP-1 plasmids, which are important in the clinical and environmental context, are highly promiscuous plasmids and were reported to often carry multiple ARGs, suggesting their important role in ARG dissemination (Popowska and Krawczyk-Balska, 2013; Wolters et al., 2015; Heuer et al., 2012). High abundance of populations carrying these plasmids seemed to be related to pollution (Smalla et al., 2006; Heuer et al., 2012). In addition, antibiotic pollution from manufacturing facilities has been shown to promote horizontal mobility of plasmids carrying ARGs among environmental bacteria (Flach et al., 2015).

In addition to plasmids, integrons are suspected to play a major role in disseminating ARGs. They are genetic platforms specialized in capturing and expressing genes in the form of gene cassettes and are usually associated with the plasmid groups mentioned above (Thorsted et al., 1998; Bahl et al., 2007; Eikmeyer et al., 2012; Heuer et al., 2012; Gaze et al., 2013). In addition, they frequently contain arrays of gene cassettes which can be excised and incorporated in new genetic contexts within a genome or between cells via MGEs (Stokes and Hall, 1989). Class 1 integrons are the best studied integrons which are minimally constituted of an *intI1* gene encoding a site-specific recombinase and of a recombination site *attI* where the integrase IntI1 catalyzes the insertion of gene cassette. They are not only restricted to the clinical context, but also widespread in different environments, particularly those exposed to antibiotics (Gaze et al., 2011; Heuer et al., 2011; Kristiansson et al., 2011; Moura et al., 2012; Li et al., 2009, 2010).

In recent years industrial discharges from antibiotic manufacturing have raised concerns since such discharge is often not controlled, but has been recognized as a risk factor for fostering AR development and dissemination (Larsson, 2014; Flach et al., 2015; Bielen et al., 2017; González Plaza et al., 2018). High, mg/L-levels of antibiotics, particularly fluoroquinolones, tetracycline or penicillins, have been detected in effluents from manufacturing facilities in countries like India, China and Korea (Larsson, 2014), underlying the importance of studying the consequences of this strong selection pressure on exposed microbial communities. Such industrial discharges that polluted receiving aquatic environments are assumed to contribute to an enrichment of ARGs and MGEs facilitating their spread, such as integrons, transposons and plasmids (Li et al., 2009, 2010; Kristiansson et al., 2011; Flach et al., 2015). Enriched ARGs represented several classes of resistance mechanisms even though only one single antibiotic class was measured at high levels at each site (Li et al., 2010; Kristiansson et al., 2011). It was therefore hypothesized that the enrichment of diverse ARGs was due to co-resistance that may be genetically linked, in combination with an increased frequency of HGT events.

However, unacceptable practices of discharging hazardous waste from antibiotic manufacturing are not restricted to Asian countries. We have recently investigated effluents from two Croatian pharmaceutical industries that were involved in synthesis of macrolide antibiotic azithromycin (AZI) or formulation of different veterinary drugs, including sulfonamides and tetracyclines. We showed high concentrations of macrolides (mg/L) and high levels of corresponding macrolide ARGs in effluents of AZI-production and in the recipient Sava river sediments (Bielen et al., 2017; Milaković et al., 2019). In contrast, lower levels of sulfonamides and oxytetracycline (μg/L) were detected in effluents from a formulation industry (Bielen et al., 2017). Antibiotic exposure may induce recruitment of ARGs from effluent-receiving sediments to human and animal pathogens by HGT; however, the potential for plasmid-mediated spread of ARGs in environments polluted by industrial discharges has rarely been studied (Flach et al., 2015).

The present study thus aimed to comprehensively explore the extent to which industrial discharges from two antibiotic production facilities in Croatia impact the promotion and spread of AR. We used quantitative real-time PCR to determine the relative abundance of MGEs such as BHR IncP-1 plasmids (*korB*) and class 1 integrons (*intI1*) as well as various resistance genes encoding sulfonamide (*sul1*, *sul2*), quaternary ammonium compound (*qacE/qacEΔ1*) and tetracycline resistance [*tet*(A)] in effluents and receiving river and creek sediments. Plasmid-mediated spread of resistance from effluent and sediment bacteria into *E. coli* recipient was investigated by exogenous plasmid isolation and captured plasmids were characterized with respect to their replicon type, presence of ARGs and integrons and AR phenotype.

2. Material and methods

2.1. Sampling

We used the same effluent samples of two Croatian pharmaceutical industries situated in the northwest of Croatia as described recently (Bielen et al., 2017; González Plaza et al., 2018; Milaković et al., 2019). Briefly, industrial facility 1 manufactures the macrolide antibiotic azithromycin through synthesis from another macrolide, erythromycin and discharges its effluent into the Sava river, near the city of Zapresic. Industrial facility 2 formulates veterinary drugs including sulfonamide, tetracycline, fluoroquinolone and beta-lactam antibiotics, and discharges its effluent into the nearby creek. Effluent from industrial facility 1 was collected as grab sample from the discharge pipe, and effluent from industrial facility 2 consisted of a 24 h composite sample. Both effluent samples were collected in sterilized screw cap bottles (2 L) and kept at +4 °C. Immediately upon return to the laboratory, aliquots of 50–100 mL were vacuum-filtered through a 0.22 μm pore-size membrane (GE Healthcare Life Sciences) to collect the bacterial cells, and filters were then stored at −80 °C until DNA extraction. In addition, cells for biparental mating assays were collected from 1 mL of each effluent by centrifuging for 5 min at 11,000 ×*g*. After discarding the supernatant, the remaining cell pellets were resuspended in 10% TSB containing 15% glycerol and stored at −80 °C.

Sediment samples were collected from the Sava river and creek at the same time as effluent samples (January and February 2016). Sava river sediments were taken at the discharge site near the city of Zapresic (DW0), one site upstream of (UP, 7500 m) and 3 sites downstream of discharge point (DW300, 300 m; DW700, 700 m and DW4500, 4500 m) (Fig. 1). The sites DW300 and DW700 are solely under the impact of discharges from industrial facility 1. Site DW700 is located just upstream of where the small river Krapina, which receives effluents from wastewater treatment plant of the city of Zapresic, enters the Sava river. These municipal effluents thus influence the site DW4500, which is located downstream from the Zapresic, in the Zagreb city area (ca. 1 million inhabitants). Sampling site UP, situated upstream of discharge point, close to Slovenian border, represented reference site which was most likely not subjected to any significant anthropogenic antibiotic pollution. Sediment samples of the creek were collected at the discharge site of effluents from industrial facility 2 (DW0), one site upstream (UP, 300 m) and one site downstream (DW 3000, 3000 m) of effluent discharge. Site UP served as a background control. Both UP and DW0 sites are located in agricultural area, while DW300 site is located in forested area.

From each site, four sub-replicates (top 10 cm) were collected within approximately 1–2 m apart using the plastic core tube. Collected sediment samples were stored in sterile plastic bags; each replicate was about 500 g (fresh weight). Immediately upon return to the laboratory, aliquots of sediments (5 g) were stored at −80 °C until DNA was extracted. Composite sediment samples (10 g of each replicate) were also prepared and used for immediate culturing or stored at −80 °C for biparental mating assays after suspension of 1 g of sediment in 9 mL of 10% Tryptic Soy Broth (TSB; BD, Franklin Lakes, NJ, USA) containing15% glycerol (v/v). The remaining of the composite sediment samples were air-dried at ambient temperature for subsequent chemical analyses of antibiotics.

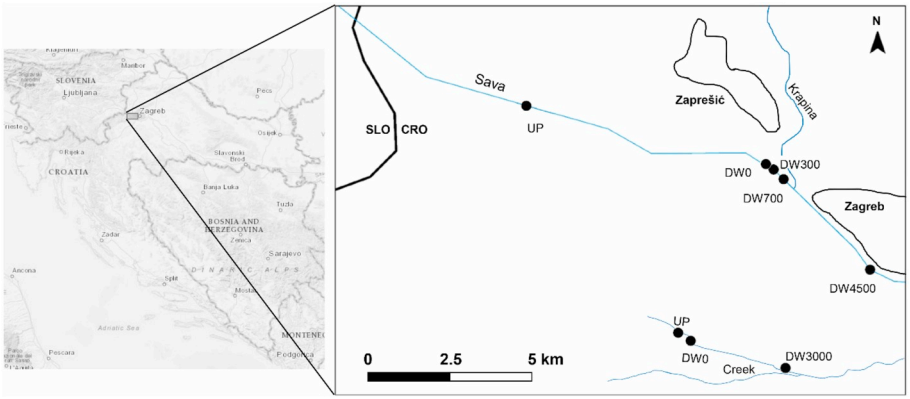

Fig. 1. Location of sampling sites in the Sava river and creek analyzed in this study.

2.2. Chemical analyses of antibiotics

The sulfonamide antibiotics (sulfadiazine and sulfamethazine) were extracted from creek sediments using pressurized liquid extraction and subsequently analyzed by reversed-phase liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS) as previously described (Senta et al., 2008, 2013). The concentration data presented for Sava river sediments are based on previous analysis of macrolide antibiotics in those sediment samples (Milaković et al., 2019).

2.3. Culturing bacteria from sediments

One gram of each composite sediment sample was suspended in sterile saline (0.85% NaCl) by vortexing. To enumerate total bacteria, serial 10-fold dilutions were cultured on three replicate R2A agar plates containing cycloheximide (CYC; 100 mg/L, Sigma, Steinheim, Germany). To enumerate resistant bacteria, serial dilutions were cultured on the same plates supplemented with azithromycin (AZI; 15 mg/L) (Fluka, Germany) for river sediment samples or oxytetracycline (OTC; 25 mg/L) (Sigma, Steinheim, Germany) for creek sediment samples. Colony forming units (CFU) were counted after a five-day incubation incubation at 28 °C.

2.4. Extraction of total community DNA from sediments and effluents and quantification of target genes

Total community (TC-) DNA was extracted directly from sediment samples and filters using the Power Soil DNA isolation kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's recommendations. Quantitative real-time PCR (qPCR) 5′-nuclease assay was conducted with extracted TC-DNA to quantify *korB* as gene marker for IncP-1 plasmids, class 1 integron-integrase gene *intI1*, quaternary ammonium compound resistance gene *qacE/qacEΔ1*, tetracycline resistance gene *tet*(A), sulfonamide resistance genes *sul1* and *sul2* as well as bacterial 16S rRNA genes. The primers and probes targeting these genes and qPCR conditions are listed in Table 1. To adjust for differences in bacterial DNA extraction and amplification efficiency between samples, the relative gene abundance was calculated using the following equation: $\log_{10} 2^{\wedge(\text{Ct 16S rRNA gene} - \text{Ct target gene})}$. All qPCR assays were performed in a CFX96 real-time (RT) PCR detection system (Bio-Rad, Hercules, USA). Differences between relative abundance of target genes at each DW site and reference UP site were tested for significance by Kruskal-Wallis test ($p < 0.05$).

2.5. Exogenous plasmid isolation through biparental assay

The kanamycin- and rifampicin-resistant *Escherichia coli* CV601 *gfp* +, carrying the *gfp* gene coding for green fluorescence protein (GFP) (Heuer et al., 2002; Wolters et al., 2015) was used as recipient strain for biparental exogenous plasmid capturing as described previously (Binh et al., 2007). Briefly, the recipient strain was cultured overnight in TSB supplemented with kanamycin (KAN; 50 mg/L), and rifampicin (RIF; 50 mg/L) at 28 °C. Cells were pelleted by centrifugation, washed (three times) and finally resuspended in 1 mL of 0.1 X TSB. Defrosted sediment or effluent samples were shaken for 2 h in 0.1 X TSB at 28 °C. Large particles were settled out, 3.5 mL of suspension was mixed with 50 µL of *E. coli* cells. This mixture was centrifuged, washed three times in 0.1 X TSB and transferred to a membrane filter placed on plate count agar (PCA; Merck; Darmstadt, Germany) supplemented with CYC (100 mg/L). Controls with only recipient or sediment bacteria were also prepared as described above. After overnight incubation at 28 °C, the suspended mating mixtures were spread plated on PCA with CYC (100 mg/L), KAN (50 mg/L) and RIF (50 mg/L) to enumerate the total number of recipients as well as on the same plates supplemented with tetracycline (TET; 15 mg/L) (creek and river sediments) or erythromycin (ERY; 50 mg/L) (only river sediments) to enumerate transconjugants. Transconjugants were confirmed by fluorescence emission and BOX-PCR fingerprints (Martin et al., 1992). Transfer frequencies were calculated based on the following formula:

Transfer frequency:CFU mL^{-1} of transconjugants/CFU mL^{-1} of recipients.

2.6. Isolation and digestion of plasmid DNA

Plasmid DNA was isolated from transconjugants using the QIAprep miniprep buffers (Qiagen, Hilden, Germany) and chloroform extraction followed by isopropanol precipitation (Wichmann et al., 2014). Plasmid DNA was digested with restriction enzyme *Not*I (Thermo Fisher Scientific, Waltham, MA, USA), analyzed on a 1% agarose gel and grouped into restriction profile patterns according to the number of different-sized fragments.

2.7. Characterization of captured plasmids by end-point and RT-PCR as well as Southern blot analysis

In order to assign captured plasmids to known plasmid groups, plasmid DNA from transconjugants was analyzed by RT-PCR for the presence of IncP-1 (*korB*), IncP-1ε (*trfA*), IncI and IncF (*traI*) plasmids. Only IncN plasmids (*rep*) were identified by end-point PCR. In order to determine which resistance genes are present on captured plasmids, plasmid DNA was screened by RT-PCR for the presence of the same resistance genes (*qacE/qacEΔ1*, *tet*(A), *sul1* and *sul2*) and integron-integrase gene (*intI1*) as described above. Detection of sequences specific for mercury resistance operon (*merRTΔP*) was performed by end-point PCR. A summary of primer sets and end-point or RT-PCR conditions used for targeting the above mentioned genes is given in Table 1.

Plasmids harboring *korB* (IncP-1 subgroup) were additionally

Table 1
Primer systems used in this study.

Target gene/plasmid group	Primers	Sequence [5′-3′]	Annealing temp.	Reference
korB (IncP-1)	korB-F	TCATCGACAACGACTACAACG	54 °C	Jechalke et al., 2013a,b
	korB-Fz	TCGTGGATAACGACTACAACG		
	korB-R	TTCTTCTTGCCCTTCGCCAG		
	korB-Rge	TTYTTCYTGCCCTTGGCCAG		
	korB-Rd	TTCTTGACTCCCTTCGCCAG		
	tp_korBgz	TSAGGTCGTTGCGTTGCAGGTTYTCAAT		
	tp_korB	TCAGYTCRTTGCGYTGCAGGTTCTCGAT		
traI (IncI1)	IncI1_traI_Fwd	TTCTTCTTCCCCTACCATC	60 °C	Blau et al., 2018
	IncI1_traI_Rev	CATTTTCCAGCGTGTTTC		
	IncI1_traI_TP	CGGCTTTTCACTTCGTGGTT		
traI (IncI2)	IncI2_traI_Fwd	CAAGAACAGAAACAGGCA	60 °C	Blau et al., 2018
	IncI2_traI_Rev	TCCCGCAGATAACAGATA		
	IncI2_traI_TP	CCAAACCAACCACAACCA		
traI (IncF)	682_F	CACGGTATGTGGGARATGCC	60 °C	Blau et al., 2018
	1073_R	TCCGGCGGCAGYATVCCRAC		
	973_P	CAGCAGGCGGTGRCRCAGGC		
trfA (IncP-1ε)	941f	ACGAAGAAATGGTTGTCCTGTTC	60 °C	Bahl et al., 2009; Dealtry et al., 2014
	1014r	CGTCAGCTTGCGGTACTTCTC		
	tp_trfAε_965	CCGGCGACCATTACAGCAAGTTCATTT		
rep (IncN)	rep-1	AGTTCACCACCTACTCGCTCCG	55 °C	Götz et al., 1996
	rep-2	CAAGTTCTTCTGTTGGGATTCCG		
intI1	intI1-LC1	GCCTTGATGTTACCCGAGAG	60 °C	Barraud et al., 2010
	intI1-LC5	GATCGGTCGAATGCGTGT		
	intI1-P	ATTCCTGGCCGTGGTTCTGGGTTTT		
tet(A)	tetA-qfw	CCGCGCTTTGGGTCATT	60 °C	Guarddon et al., 2011
	tetA-qrv	TGGTCGCGTCCCAGTGA		
	q-tetA-P	TCGGCGAGGATCG		
qacE and/or *qacEΔ1*	qacEall-F	CGCATTTATTTTCTTTCTCTGGTT	60 °C	Jechalke et al., 2014
	qacEall-R	CCCGACCAGACTGCATAAGC		
	qacEall-P	TGAAATCCATCCCTGTCGGTGT		
sul1	q-sul_1 653f	CCGTTGGCCTTCCTGTAAAG	60 °C	Heuer and Smalla, 2007
	q-sul_1 719r	TTGCCGATCGCGTGAAGT		
	tp_sul1	CAGCGAGCCTTGCGGCGG		
sul2	q_sul2 595f	CGGCTGCGCTTCGATT	60 °C	Heuer and Smalla, 2007
	q_sul2 654f	CGCGCGCAGAAAGGATT		
	tp_sul2 614	CGGTGCTTCTGTCTGTTTCGCGC		
merRTΔP	merRT-P-P	GGGAGATCTAAAGCACGCTAAGGCRTA	62 °C	Osborn et al., 1993
	merRT-P	GGGGAATTCTTGACWGTGATCGGGCA		
16S rRNA gene	Bact1369F	CGGTGAATACGTTCYCGG	56 °C	Suzuki et al., 2000
	Prok1492R	GGWTACCTTGTTACGACTT		
	TM1389F_P	CTTGTACACACCGCCCGTC		

screened by Southern hybridization with probe for IncP-1ε or with a mixed IncP-1 probe targeting subgroups α, β, γ, δ, and ε. Plasmid DNA digestion was performed as described above. Digested plasmids were separated on a 1% agarose gel and then transferred to a positively charged nylon membrane (Roche, Mannheim, Germany). Southern blot hybridization was carried out with digoxigenin-labelled probe generated from PCR amplicons obtained with reference plasmids pKJK5 for IncP-1ε and R751 for IncP-1β (Bahl et al., 2009; Dealtry et al., 2014). The recipient strain and one of the plasmids negative for *korB* were used as negative controls.

2.8. Antibiotic susceptibility testing

A representative subset of transconjugants was analyzed for resistance to tetracycline, trimethoprim, doxycycline, ampicillin, amoxicillin, chloramphenicol, streptomycin, cefotaxime, ciprofloxacin, ceftriaxone, gentamycin, nalidixic acid, ceftazidime and sulfadiazine using disk diffusion method (Wolters et al., 2015). The recipient *E. coli* CV601 was included as a negative control. The minimal inhibitory concentration (MIC) of sulfamethoxazole for the recipient and transconjugants was determined using Etest strip (Biomerieux, France) according to instructions from the provider. The MICs of erythromycin and azithromycin were performed by broth microdilution as described previously (Donato et al., 2010). MIC values that were 2–3 fold increased compared with those for the recipient are designated as moderate resistance while those that were at least 4-fold increased in comparison with the recipient are designated as high resistance.

3. Results

3.1. Antibiotic pollution of sediments

The sediments taken from Sava river at effluent discharge site (DW0) were highly contaminated by macrolide antibiotics characteristic for corresponding manufacturing facility - azithromycin as a final product and erythromycin as a precursor in the synthesis (Table 2). The total concentration of these two macrolides reached a high level of around 10 mg/kg at DW0 and decreased with distance further downstream but was still high (> 1 mg/kg) at sites DW300 and DW700. Low total concentrations of macrolides (< 5 μg/kg) were detected in river sediments sampled upstream of discharge (UP).

For the analysis of sediments taken from the creek, the sulfonamide antibiotics were chosen as the unique targets because they usually dominate, together with oxytetracycline, in effluents of industrial facility 2 (Bielen et al., 2017). Oxytetracycline, a tetracycline antibiotic, was not analyzed because quantitative method for the analysis of tetracyclines in sediment compartment was not established in our lab; however, accumulation of tetracyclines is expected due to their constant input through effluent discharge and the strong sequestration to organic matter (Huang et al., 2011). The lowest total concentration of sulfonamides (sulfadiazine and sulfamethazine) was found at UP creek site (39 μg/kg) and an around 10-times higher concentration at site

Table 2
Average antibiotic concentrations in the sediment samples analyzed.

Industrial facility	Recipient water body	Sampling site	Antibiotics (μg/kg dry sediment)
			Total macrolides[a,b]
1 (AZI-synthesis)	Sava river	UP	4.60
		DW0	10,176
		DW300	2710
		DW700	1253
		DW4500	258

Industrial facility	Recipient water body	Sampling site	Antibiotics (μg/kg dry sediment)
			Total sulfonamides[c]
2 (drug-formulation)	Creek	UP	39
		DW0	453
		DW3000	1175

Sampling sites: UP, upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW3000, 3000 m downstream; DW4500, 4500 m downstream.
[a] Sum of azithromycin and erythromycin.
[b] Milaković et al. (2019).
[c] Sum of sulfadiazine and sulfamethazine.

DW0. However, the largest amounts of sulfonamides were not found at site DW0 but at more distant site DW3000 (> 1 mg/kg).

3.2. Increased and transferable antibiotic resistance in industrially-impacted river and creek sediments

Culture-based analysis of resistant bacteria from Sava river sediments impacted by pollution from AZI-production (industrial facility 1) showed a significantly higher proportion of AZI-resistant bacteria in sediments at discharge (DW0) and downstream sites (DW300 - DW4500) compared with upstream (UP) site (Kruskal-Wallis test, $p < 0.05$; Fig. 2a). For analysis of culturable resistant bacteria from creek sediments, OTC rather than sulfonamide antibiotic was used because it is one of the drugs produced by industrial facility 2 and often found at increased μg/L concentrations in industrial effluents and surface water of the receiving creek (Bielen et al., 2017). Additionally, tetracycline and sulfonamide resistance is frequently linked in aquatic bacteria (Hu et al., 2008; Kim et al., 2008; Nonaka et al., 2012). Similar to river sediments, culturing showed a considerably higher proportion of OTC-resistant bacteria in effluent-receiving creek sediments, both at DW0 and downstream DW3000 site compared with UP site (Kruskal-Wallis test, $p < 0.05$; Fig. 2b). As expected, highest proportions of cultivable resistant bacteria were found in sediments at both DW0 sites (AZI-resistant bacteria 67%, OTC-resistant bacteria 31%) followed by a sharp decrease further downstream (Fig. 2a, b).

Exogenous plasmid isolations from the river bacteria in sediment samples taken along a gradient of macrolide pollution into *E. coli* CV601 were performed in order to capture transferable plasmids conferring ERY- or TET-resistance. TET was used for selection of captured plasmids because typically no intrinsic resistance is observed. ERY-resistant transconjugants were obtained from three sites, i.e. UP, DW0 and DW700 (Fig. 2c). Transfer frequencies were considerably higher for both polluted sites (DW0 and DW700) in comparison with non-polluted UP site. However, higher log transfer frequency was obtained for more

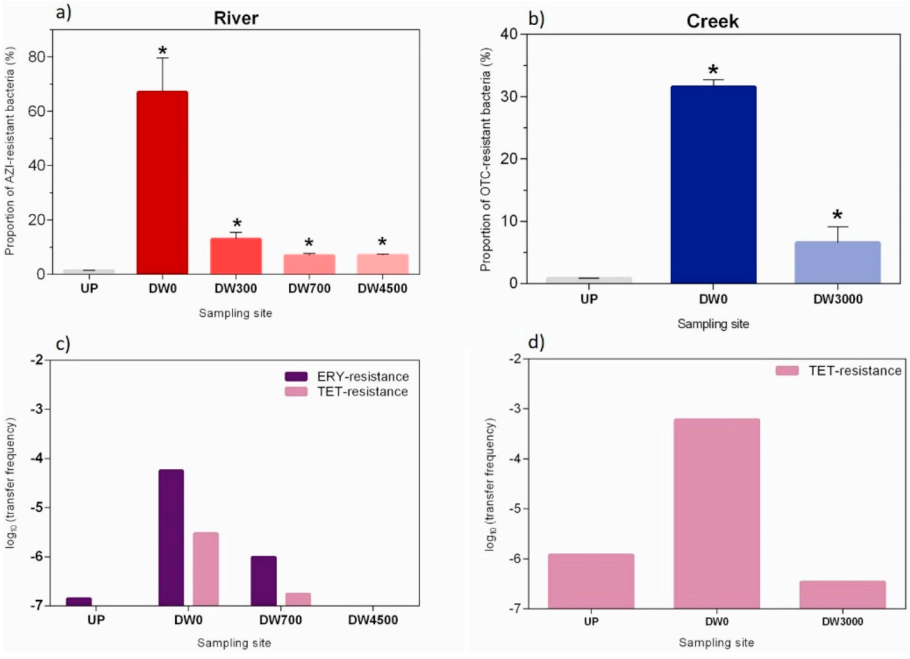

Fig. 2. The proportion of cultivable bacteria (mean ± SD) resistant to azithromycin (AZI) in Sava river sediments (a) and to oxytetracycline (OTC) in creek sediments (b). Transfer frequencies of erythromycin (ERY)- and tetracycline (TET)-resistance from Sava river sediments (c) and TET-resistance from creek sediments (d). Significant differences in the percentage of resistant bacteria were assessed by Kruskal-Wallis test ($p < 0.05$). UP - upstream site, DW0 - discharge site, DW300 - 300 m downstream of discharge, DW700 - 700 m downstream, DW3000 - 3000 m downstream, DW4500 - 4500 m downstream.

polluted site DW0 (−4.3) than for DW700 (−6.0). Further, both DW0 and DW700 sites showed considerably higher log transfer frequencies of TET-resistance plasmids (DW0, −5.5; DW700, −6.8) compared with UP or DW4500 site from which no TET-resistant transconjugants were obtained (Fig. 2c). Additionally, no transconjugants could be captured from effluent of industrial facility 1.

Besides river sediments, transferable TET-resistance plasmids could be also captured from creek sediments from sites UP, DW0 and DW3000 with log transfer frequencies of −5.9, −3.2, and −6.5 (Fig. 2d). However, only DW0 site showed higher transfer frequency in comparison with UP site. In addition to sediments, TET-resistant transconjugants were also obtained from effluent of industrial facility 2 at log transfer frequency of −4.74.

3.3. Transferable plasmids belonged to IncP-1 and IncN groups and conferred multiple resistances

A total of 153 potential transconjugants were further confirmed based on their growth on selective media, GFP fluorescence and BOX-PCR. PCR-based screening of the transconjugants for the presence of *korB*, *traI*, *trfA*, *rep*, *intI1*, *qacE/qacEΔ1*, *sul1*, *sul2*, *tet*(A) and *merRTΔP* genes revealed that the plasmids captured formed 22 different groups (Table S1).

The majority of exogenously captured plasmids (88/153) were assigned to the IncP-1 group as revealed by qPCR targeting the *korB* gene. Further characterization by RT-PCR targeting the *trfA* gene and Southern blot hybridization with primers/probes specific for IncP-1ε showed that majority of IncP-1 plasmids belonged to this subgroup. These IncP-1ε plasmids originated from both study areas including effluent of industrial facility 2 (WW2) and creek sediments from both upstream and discharge site (UP, DW0) as well as river sediments closer to discharge pipe from industrial facility 1 (DW0 and DW700) (Tables S1 and 3). Furthermore, all of these IncP-1ε plasmids, except one (#5), were tested positive for integrase gene of class 1 integrons (*intI1*), the TET-resistance gene *tet*(A), the sulfonamide resistance gene *sul1* and the quaternary ammonium compounds resistance gene *qacE/qacEΔ1* (Table 3). In addition, based on the antibiotic susceptibility testing, all plasmids of the IncP-1ε group conferred resistance to antibiotics from 2 to 4 antibiotic classes (Table 3).

Two transconjugants (Table S1) contained IncP-1β plasmids that were captured from river sediment from polluted DW700 site. This plasmid did not carry any of the tested genes but displayed resistance toward macrolides according to antibiotic susceptibility testing (#12; Table 3). One transconjugant (#6) carried an IncN plasmid and originated from creek sediment from discharge site. This transconjugant conferred resistance to multiple antibiotic classes including tetracyclines, trimethoprim, sulfonamides and beta-lactams (Table 3). The plasmids contained in the remaining transconjugants could not be assigned to any of the plasmid group tested (Tables S1 and 3). The IncN plasmid was the only one that harbored *sul2* in addition to *qacE/qacEΔ1*, *sul1* and *tet*(A) genes (Tables S1 and 3).

The number of resistances to different antibiotic classes captured

Table 3
Plasmid specific sequences and resistance genes detected via end-point PCR, Southern blot hybridization and RT-PCR in plasmid DNA of *E. coli* CV601 transconjugants and corresponding antibiotic resistance patterns as determined by antibiotic susceptibility testing.

#	Site and industrial facility[a]	Antibiotic for capturing	Inc groups	Resistance and integrase genes	Antibiotic resistance profile[b]
1	UP[1]	ERY	IncP-1	*intI1*, *qacE/qacEΔ1*, *merRTΔP*, *sul1*	SMX, AZI, ERY
2	DW0[1]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP[M], SMX[M], DOX[M]
3	DW0[1]	ERY	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AZI, ERY, DOX[M]
4	DW0[1]	ERY	IncP-1	*intI1*, *qacE/qacEΔ1*, *sul1*	SMX, ERX, AZI
5	DW0[1]	TET	IncP-1ε	*intI1*, *sul1*, *tet*(A)	TET, SMX, DOX[M]
6	DW0[1]	TET	IncN	*intI1*, *qacE/qacEΔ1*, *sul1*, *sul2*, *tet*(A)	TET, TMP, SMX, AM, AMX, DOX[M]
7	DW0[1]	ERY		*intI1*, *qacE/qacEΔ1*, *sul1*	AM, AMX, AZI, ERY, SMX
8	DW0[1]	TET			TET
9	DW0[1]	ERY	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AZI, ERY, DOX[M]
	DW0[1], DW700[1]	TET			
10	DW700[1]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, DOX, ERY
	DW0[1,2]				
11	DW700[1]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX
12	DW700[1]	ERY	IncP-1β		ERY, AZI[M]
13	DW700[1]	ERY		*sul2*	SMX, AZI, ERY
14	DW700[1]	ERY		*sul2*	SMX, AZI, ERY
15	DW700[1]	TET			TET, DOX
16	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX[M]
17	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, DOX
18	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TMP, SMX, TET[M], DOX[M]
19	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX
20	UP[2]	TET		*tet*(A)	TET
21	UP[2]	TET		*tet*(A)	TET
22	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	DOX, SMX, TET[M]
23	UP, DW0[2]	TET		*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX[M]
24	DW0[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AM, AMX, DOX
25	DW0[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AM, AMX, DOX
26	DW0[2]	TET		*tet*(A)	TET, DOX[M]
27	DW3000[2]	TET		*merRTΔP*, *sul2*	AM, AMX, DOX, CHL
28	DW3000[2]	TET		*sul2*	AM, AMX, DOX, CHL
29	DW3000[2]	TET		*sul2*	AM, AMX, DOX, CHL
30	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	SMX, TET[M], DOX[M]
31	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX[M]
32	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX[M]
33	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, DOX, SMX, ERY[M]

[a] UP, upstream; DW0, discharge; WW2, effluent from drug-formulation facility; DW3000, 3000 m downstream of discharge; DW700, 700 m downstream of discharge; 1, industrial facility 1 - AZI-synthesis; 2, industrial facility 2 - drug-formulation.

[b] TET: tetracycline; ERY: erythromycin; AZI: azithromycin; TMP: trimethoprim; DOX: doxycycline; AM: ampicillin; AMX: amoxicillin; CHL: chloramphenicol; S: streptomycin; SMX: sulfamethoxazole; M - moderate antibiotic resistance.

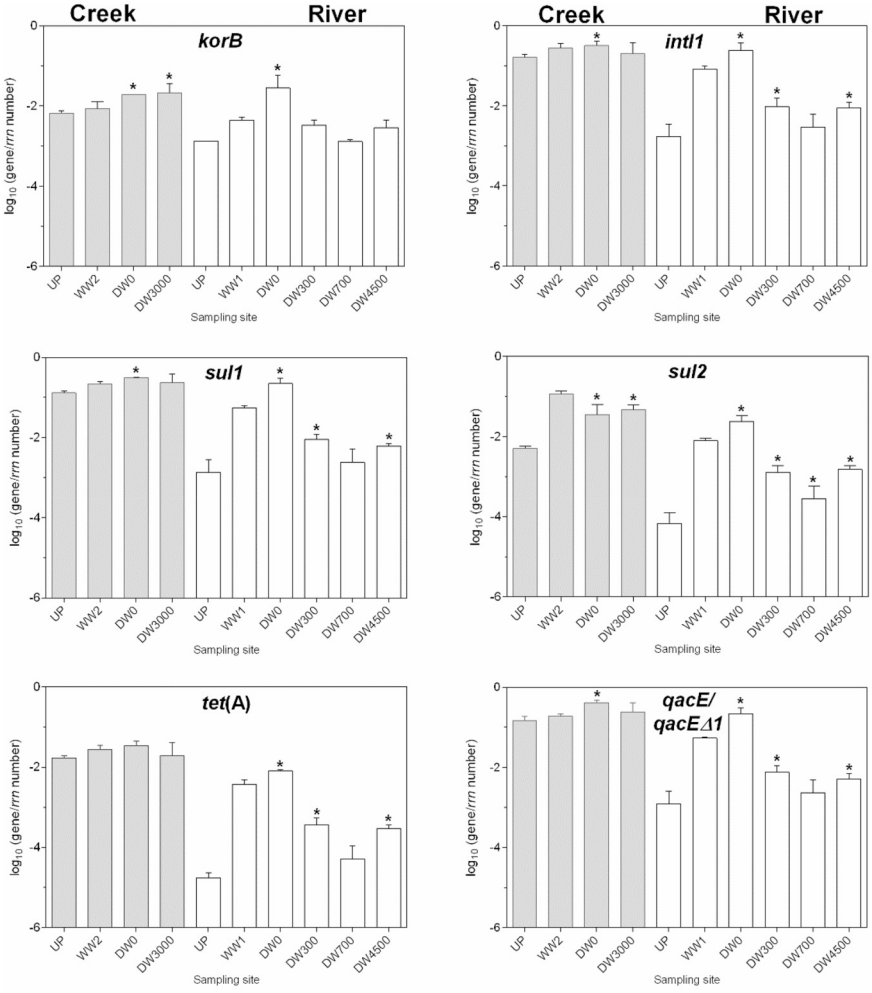

Fig. 3. Relative abundance of antibiotic- and disinfectant-resistance genes [*sul1*, *sul2*, *tet*(A), *qacE/qacEΔ1*], class 1 integron-integrase gene (*intI1*) and IncP-1 plasmids (*korB*) in industrial effluents (WW1 and WW2) and sediments of receiving river and creek from different sites. WW1 - effluent from azithromycin-synthesis facility. WW2 - effluent from drug-formulation facility. Each value is the mean ± SD of four replicates. Sediment sampling locations: UP, upstream; DW0 - discharge; DW300 - 300 m downstream of discharge; DW700 - 700 m downstream; DW3000 - 3000 m downstream and DW4500 - 4500 m downstream. Asterisks represent significant difference ($p < 0.05$; Kruskal-Wallis) between each DW site and reference UP site.

tended to be higher for transconjugants from most polluted sites (Sava river, DW0 site; creek, DW3000 site) in comparison with reference UP sites (Tables 1 and S1). Strikingly, all plasmids captured from the creek at the site situated 3 km downstream of discharge (DW3000), displayed the most pronounced multi-resistance phenotypes as they conferred resistance against five antibiotic classes including tetracyclines, trimethoprim, sulfonamides, beta-lactams and chloramphenicol.

3.4. Effects of industrial effluent discharges on the relative abundance of target resistance genes and MGEs in receiving sediments

We quantified the abundance of MGEs such as IncP-1 plasmids (*korB*) and class 1 integrons (*intI1*), and their typically associated resistance genes [*sul1*, *qacE/qacEΔ1* and *tet*(A)] as well as the sulfonamide resistance gene *sul2* in TC-DNA from two industrial effluent samples as well as sediment samples of the receiving river and creek by using qPCR (Fig. 3).

TC-DNA from effluent of industrial facility 1 (WW1) contained high relative abundances of resistance genes [*sul1*, *sul2*, *tet*(A) and *qacE/qacEΔ1*] with around −1 to −2 log gene copies/*rrn* copies (Fig. 3). In addition to resistance genes, TC-DNA from WW1 effluent contained high levels of MGE genes, *intI1* (−1 log gene copies/*rrn* copies) and *korB* (−2 log gene copies/*rrn* copies). The discharge of these effluents significantly increased the relative abundances of *sul1, sul2, tet*(A) and *qacE/qacEΔ1* genes in sediments from the receiving river at nearly all downstream (DW) sites compared with the UP site ($p < 0.05$; Kruskal-Wallis; Fig. 3). The highest relative abundances of the above genes occurred at discharge DW0 site (−0.6 to −2 log gene copies/*rrn* copies), with the highest relative abundance for *qacE/qacEΔ1* and *sul1*. At all downstream sites (DW300 - DW4500) the relative abundances of target resistance genes were around −2 to −3 log gene copies/*rrn* copies (Fig. 3). Similarly, the relative abundance of the *intI1* gene was also significantly increased in river sediments downstream vs upstream and its relative abundance was similar to that of *qacE/qacEΔ1* and *sul1*. However, the relative abundance of *korB* was higher only at DW0 site in comparison with UP site.

In effluent of industrial facility 2, all analyzed resistance and MGE genes (*korB* and *intI1*) were also detected in high levels (up to −0.6 log gene copies/*rrn* copies; Fig. 3). However, the discharge of these effluents only slightly, but significantly, increased the relative abundances of all target genes, except *tet*(A), in receiving creek sediments compared to sediment from the UP site ($p < 0.05$; Kruskal-Wallis; Fig. 3). The abundances of *intI1*, *sul1* and *qacE/qacEΔ1* genes relative to the *rrn* copy number were significantly higher only at DW0 site, with the average −0.5 log gene copies/*rrn* copies. Compared to UP site, the relative abundances of *korB* and *sul*2 genes were significantly higher at both DW0 and DW3000 sites with −1.4 log units (*korB*) and − 1.7 log units (*sul2*), respectively. However, strikingly, compared to river sediment from UP site, relative abundances of all target genes were up to 3 orders of magnitude higher in the creek sediment samples from UP site, indicating a higher background level of ARGs in the creek than in the river.

4. Discussion

Discharges from antibiotic manufacturing facilities have repeatedly been shown to provide conditions for the selection, spread and persistence of AR in the receiving aquatic environment (Larsson, 2014; Flach et al., 2015). Our previous study demonstrated that effluents from two Croatian antibiotic manufacturing industries studied here contained high levels of organic compounds, nutrients and antibiotics (mg/L range) as well as a high proportion of ARB (Bielen et al., 2017). The present study provided further evidence that as a result of effluent discharges from these industrial facilities the proportion of ARB in effluent-receiving sediments was significantly increased compared to those in non-exposed sediment samples. This indicated either propagation of indigenous sediment bacteria due to antibiotic selection pressure or survival of ARB released with the effluent. In addition, a combination of these contributors may take place as well. Our findings are in accordance with previously published studies demonstrating increased levels of ARB in aquatic environments exposed to discharges from antibiotic manufacturing (Li et al., 2010; Flach et al., 2015; González Plaza et al., 2018).

Such increased abundance of ARB in the environment might contribute to increased levels of AR in human and animal pathogens by HGT. We have, therefore, applied exogenous plasmid capture using sediment bacteria as donor cells and GFP-marked *E. coli* CV601 recipient to study transferability of ARGs. The experiments showed that effluent discharges had an impact on the transfer of resistance, but differences between two investigated study areas were observed. In Sava river sediments, the higher transfer frequency was observed for sediments taken from macrolide-polluted sites (DW0, DW700) compared to those from least-polluted (UP) or less-polluted sites (DW4500). These observations suggest that polluted sites, particularly site DW0, may be hot-spots for plasmid-mediated transfer of ERY- and TET-resistance among the sediment bacterial population. This is likely due to the high number of bacteria as well as high nutrient and macrolide levels in sediments from these sites (Milaković et al., 2019). This is in accordance with a study by Flach et al. (2015) who showed that Indian lake sediments polluted by extreme fluoroquinolone levels through manufacturing discharges displayed higher transfer frequencies of AR plasmids compared with non-polluted sediments. They reported transfer frequencies in the range from 10^{-4} to 10^{-6} which were comparable to those obtained in this study (range from 10^{-3} to 10^{-6}). In contrast to river sediments, transfer frequency was only increased in creek sediments taken from more sulfonamide-polluted site DW0, but not from the most polluted site DW3000 in comparison with UP site. However, selection of acquired resistances was done for TET rather than for sulfonamides to avoid false positives due to high levels of intrinsic sulfonamide resistance. In addition to sediments, transconjugants were also obtained from effluent of drug-formulation facility, indicating that effluent is a source of transferable AR. We have previously reported that this effluent contained 17 μg/L of oxytetracycline, an analogue of tetracycline (Bielen et al., 2017). Additionally, previous studies have demonstrated that TET concentration as low as 10 μg/L promoted HGT (Kim et al., 2014; Jutkina et al., 2016). Together, this suggests that OTC in studied effluent could stimulate plasmid-mediated HGT among effluent bacteria. To the best of our knowledge, this is one of the first reports on the transferability of ARGs harbored in bacteria resident in effluents from pharmaceutical industries. In contrast to drug-formulation effluent, no transfer of ERY- or TET-resistance to *E. coli* CV601 was obtained from effluent of AZI-synthesis facility. This is likely a consequence of very high concentrations of macrolides found in that effluent sample (6.5 mg/L total) (Bielen et al., 2017) which could have inhibited the recipient strain. Indeed, a previous study showed that the macrolide antibiotic ERY inhibited plasmid-mediated resistance transfer at 1 mg/L and even more at 10 mg/L (Jutkina et al., 2018). In contrast to AZI-production effluent, sediment bacteria from both macrolide-polluted sites (DW0 and DW700) with up to 10 mg macrolides/kg sediment successfully transferred resistance plasmids to *E. coli* CV601; however, due to sorption of macrolides to sediment, their bioavailability might be lower than the total concentration measured in sediments.

A large proportion (84/153) of the plasmids captured during this study belonged to the IncP-1ε subgroup (Table S1). The majority of them (68/84) originated from antibiotic-polluted matrices (drug-formulation effluent and creek sediment from DW0 site; river sediments from DW0 and DW700 sites). This study suggests that IncP-1ε plasmids might substantially contribute to the local adaptation and survival of the bacterial communities in response to strong pollution from antibiotic manufacturing facilities. Although IncP-1 plasmids were originally discovered in clinical isolates (Datta et al., 1971), they were later observed in a wide range of different habitats including mercury-polluted river sediment, sewage, sludge and rivers (Haines et al., 2006; Smalla et al., 2006; Bahl et al., 2009; Moura et al., 2010; Heuer et al., 2012; Oliveira et al., 2012), and a correlation of IncP-1 plasmid abundance and pollution was suggested (Smalla et al., 2006). Further, in the present study, a few captured plasmids from macrolide-polluted river sediments could be assigned to the IncP-1β subgroup and IncN incompatibility group. The latter is in agreement with the study of Flach et al. (2015), who captured IncN plasmids from Indian lake impacted by extreme fluoroquinolone pollution from manufacturing sites. Considering the BHR of both IncP-1 and IncN plasmids (Musovic et al., 2006; Klümper et al., 2015; Shintani et al., 2015; Matsumura et al., 2018), we suggest that captured plasmids might be widely shared among sediment bacteria in the studied river and creek. An increased and high relative abundance of the promiscuous IncP-1 plasmids in sediments from both discharge sites (Fig. 3) further indicated that these sites are hot-spots of bacterial populations carrying this plasmid type. This is the first report on IncP-1 plasmids in antibiotic manufacturing effluents and receiving freshwater sediments. These plasmids persisted in downstream sediments as shown by *korB* qPCR data (Fig. 3) which may allow further environmental spread of ARGs localized on these plasmids. We therefore screened plasmids and investigated sediments for the presence of various resistance genes, including tetracycline [*tet*(A)], quaternary ammonium compound (*qacE/qacEΔ1*) and sulfonamide resistance genes (*sul1*, *sul2*). These resistance genes were selected for three reasons: i) all of them, except *qacE/qacEΔ1*, were previously identified in this study area by functional metagenomics (González-Plaza et al., 2018); ii) association of these genes with MGEs - the *sul1* and *qacE/qacEΔ1* genes are often found on class 1 integrons localized on IncP-1 plasmids (Wolters et al., 2015; Jechalke et al., 2014), while the *sul2* and *tet*(A) genes are often located on various transferable plasmids belonging to different incompatibility groups (Heuer et al., 2009; Flach et al., 2015; Blau et al., 2018); iii) TaqMan probe-based RT-PCR method targeting these genes has been established during our previous work. We showed that IncP-1ε plasmids captured from effluent and receiving sediments were associated with *qacE/qacEΔ1*, *sul1* and *tet*(A) resistance genes as well as class 1 integrons (*intI1*) as also shown in other studies (Jechalke et al., 2014; Wolters et al., 2015). In addition to these resistance genes, IncP-1ε and IncN plasmids characterized in this study often conferred phenotypic resistance to other clinically relevant antibiotic classes such as macrolides, trimethoprim or beta-lactams. Such multi-resistance phenotypes seemed to be the most pronounced for plasmids captured from the most polluted sediments (Sava river, site DW0; creek, site DW3000), indicating that industrial discharges might select for bacterial populations that carry multi-resistance plasmids. Earlier studies have demonstrated that the discharge of antibiotic manufacturing effluents contributes to the multidrug resistance among exposed environmental isolates (Li et al., 2009, 2010).

In addition to increased abundance of IncP-1 plasmids, the relative abundance of plasmid-associated resistance genes, i.e. *sul1*, *sul2*, *tet*(A) and *qacE/qacEΔ1* was also significantly elevated in river sediments impacted by AZI-synthesis effluents compared to upstream sediment. This implied that industrial discharges enriched the receiving river with

bacteria carrying these resistance genes likely due to deposition of effluent-associated bacteria or the propagation of indigenous sediment bacteria that are intrinsically resistant or acquired ARGs via plasmid-mediated transfer from effluent bacteria under selection pressure from antibiotics. Although only macrolide antibiotics (AZI, ERY) were detected in high concentrations in effluents (mg/L range) (Bielen et al., 2017) and in receiving sediments, the high levels of *sul1*, *sul2*, *tet*(A) and *qacE/qacEΔ1* genes could be due to co-selection via increasing HGT in response to exposure to macrolides. Indeed, these resistance genes have commonly been found on plasmids that often carry macrolide resistance genes (Nonaka et al., 2012; Dolejska et al., 2014; Rahube et al., 2014), and thus, could be selected due to the presence of macrolides in analyzed sediment samples. In addition, the trends of increased relative abundances of the *intI1*-associated class 1 integrons reflected the trends of the ARGs, which were found elevated in effluent-receiving sediments, except *tet*(A) in creek sediments. The class 1 integron-integrase gene, *intI1*, was previously proposed as a proxy for anthropogenic pollution (Gillings et al., 2015) and its plasmid localization would facilitate potential for HGT in these sediments.

Compared with river sediments, all resistance genes targeted and class 1 integrons were found considerably increased in creek sediments already at the upstream site by up to 3 orders of magnitude. This could be explained by, for example, input from activities such as agricultural runoff and discharges of household sewage into the creek which flows through an area without sanitation infrastructure. This could also be a possible explanation for a moderate pollution of upstream creek sediments by sulfonamide antibiotics. Nevertheless, direct discharge of partially treated effluents from drug-formulation facility affected slightly, but significantly, sediment levels of IncP-1 plasmids and some resistance genes targeted. These effluents have earlier been shown to contain high concentrations of sulfonamide antibiotics (up to 230 μg/L) (Bielen et al., 2017) and consequently, what is shown here, effluent discharges contributed to the accumulation of sulfonamides in receiving creek sediments. In addition to antibiotics, we showed in the present study that effluent also introduced the *sul*, *tet* and *qac* genes as well as MGEs such as class 1 integrons and IncP-1 plasmids in relatively high amounts into the creek ($> 10^{-2}$ gene copies/*rrn*, Fig. 3). Therefore, the introduction of these ARGs and MGEs, together with antibiotics present in effluents contributed to elevated levels of almost all target resistance genes and MGEs in sediment at the discharge site. However, contrary to the *intI1*, *sul1* and *qacE/qacEΔ1* genes, the relative abundance of *sul2*- and the IncP-1 plasmid-bearing bacteria were still maintained elevated at the site located 3 km downstream of discharge (DW3000) compared to UP site. Although *sul2* genes were often found on IncQ or the LowGC plasmids (Sköld, 2000; Smalla et al., 2000; Heuer et al., 2009), its increased abundance might point to a response of their hosts to sulfonamides, which were found in higher levels in sediments at DW3000 (> 1 mg/kg) compared to UP and DW0 sites (Table 2). This accumulation of sulfonamides might be the result of the slower flow rate of the creek at DW3000 than at DW0 site, which in turn might accelerate the sedimentation of antibiotics in the sediment. Interestingly, three plasmids exogenously captured from this DW3000 site could not be assigned to any incompatibility group tested, but carried *sul2* gene and displayed phenotypic resistance to five antibiotic classes, widely used in clinical settings for human therapy. Therefore, these plasmids replicating in *E. coli* may be important vectors for accumulating and spreading multiple and clinically relevant resistance genes among environmental bacteria, including pathogens.

In conclusion, this study has shown that industrial effluents from two Croatian antibiotic manufacturing facilities are significant sources of MGEs associated with ARGs, contributing to their increased levels in the sediments of receiving water bodies. Although effluents from both industries contained similar levels of targeted ARGs and MGEs, much more pronounced effects of effluent discharge on the resistance gene levels were observed for river sediments rather than for creek sediments due to much higher background levels of investigated genes in the creek. Effluent discharge sites might be considered as hot-spots of plasmid-mediated HGT, fostering the adaptation of sediment bacteria to stresses such as exposure to high concentrations of antibiotics and other toxic agents from pharmaceutical production. Plasmids of the IncP-1ε subgroup might have contributed to further dissemination of multiple ARGs to other bacteria in downstream environment. Further sequencing studies should be done to assign those plasmids which were not assigned by the RT-PCR systems used in the present study and to provide better insight into the entire accessory gene content of these plasmids as well as to reveal whether the same plasmids are present in clinically important pathogens from the surrounding areas.

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