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Pollution from azithromycin-manufacturing promotes macrolide-resistance gene propagation and induces spatial and seasonal bacterial community shifts in receiving river sediments

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ABSTRACT

Effluents from antibiotic manufacturing may contain high concentrations of antibiotics, which are the main driving force behind the selection and spread of antibiotic resistance genes in the environment. However, our knowledge about the impact of such effluent discharges on the antibiotic resistome and bacterial communities is still limited. To gain insight into this impact, we collected effluents from an azithromycin-manufacturing industry discharge site as well as upstream and downstream sediments from the receiving Sava river during both winter and summer season. Chemical analyses of sediment and effluent samples indicated that the effluent discharge significantly increased the amount of macrolide antibiotics, heavy metals and nutrients in the receiving river sediments. Quantitative PCR revealed a significant increase of relative abundances of macrolide-resistance genes and class 1 integrons in effluent-impacted sediments. Amplicon sequencing of 16S rRNA genes showed spatial and seasonal bacterial community shifts in the receiving sediments. Redundancy analysis and Mantel test indicated that macrolides and copper together with nutrients significantly correlated with community shift close to the effluent discharge site. The number of taxa that were significantly increased in relative abundance at the discharge site decreased rapidly at the downstream sites, showing the resilience of the indigenous sediment bacterial community. Seasonal changes in the chemical properties of the sediment along with changes in effluent community composition could be responsible for sediment community shifts between winter and summer. Altogether, this study showed that the discharge of pharmaceutical effluents altered physico-chemical characteristics and bacterial community of receiving river sediments, which contributed to the enrichment of macrolide-resistance genes and integrons.

1. Introduction

Since antibiotics are naturally produced by microorganisms in the environment, bacterial communities maintain a large collection of resistance genes, called the resistome (D'Costa et al., 2006; Surette and Wright, 2017). However, continuous environmental pollution by antibiotics and other selective agents, such as heavy metals, has perturbed the dynamics of natural systems (Di Cesare et al., 2016a; Martins et al., 2014). The selection pressure imposed by these pollutants has promoted the proliferation and spread of resistant populations and their resistance genes, thus creating a pool of resistance genes for pathogens to acquire (Li et al., 2017; Xu et al., 2017; Zhang et al., 2018). This acquisition of resistance genes is often facilitated by mobile genetic elements such as plasmids often carrying integrons which are therefore considered as key contributors in the dissemination of antibiotic resistance genes (ARGs) and promoters of multidrug resistance (Gillings et al., 2015; Heuer et al., 2012; Smalla et al., 2015; Zhang et al., 2011). Highly similar or even identical ARGs have been found in both

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environmental- and pathogenic bacteria (Forsberg et al., 2012; Nordmann and Poirel, 2005), emphasizing a potentially shared resistome. It has been shown that sub-inhibitory concentrations of antibiotics, high bacterial densities and increased nutrient availability facilitate plasmid-mediated horizontal gene transfer (HGT) (Jutkina et al., 2018; Rizzo et al., 2013). Elevated levels of antibiotics in the environment are also expected to increase the intensity of resistance gene exchange through plasmid- (Flach et al., 2015) or phage-mediated transfer. However, antibiotic discharges into the environment have been rarely regulated directly, although discharge limits for individual antibiotics have recently been proposed (Bengtsson-Palme and Larsson, 2016). Therefore, understanding the role of antibiotic-polluted environments in the evolution and dissemination of ARGs is crucial to develop effective and sustainable mitigation strategies to reduce the rising threat of antibiotic resistance on a global scale.

Effluents from antibiotic manufacturing plants have been reported to be a significant point source and dissemination route for antibiotics, antibiotic-resistant bacteria (ARB) and ARGs into the aquatic environment (Larsson et al., 2007; Li et al., 2008; Marathe et al., 2013; Rutgersson et al., 2014). For example, high concentrations (in the mg/L range) of oxytetracycline, lincomycin or fluoroquinolone antibiotics have been reported in treated effluents from pharmaceutical industries in China, Korea or India (Larsson, 2014). Discharges of such effluents have led to antibiotic contamination of surface, ground and drinking water bodies (Fick et al., 2009), and to an enrichment of multidrug-resistant bacteria, ARGs and elements facilitating their transfer (Flach et al., 2015; Kristiansson et al., 2011; Li et al., 2010).

Similarly to the above-mentioned reports from Asian countries, we have also recently investigated effluents from the local pharmaceutical industry, located near Zagreb in Croatia, which produces the macrolide antibiotic azithromycin (AZI). Macrolide antibiotics act as inhibitors of bacterial protein synthesis and have been used widely to combat respiratory tract infections and soft-tissue infections. They are highly potent against a wide variety of Gram-positive and Gram-negative organisms, and they are used as penicillin substitutes (Kaneko et al., 2007; Wierzbowski et al., 2005). Macrolides have recently been ranked as one of most important antibiotics for human medicine by the World Health Organization (WHO, 2017). Thus, it is important to preserve their efficacy in the treatment of human infections. Our chemical analyses of effluents from AZI production sites revealed very high concentrations of macrolides (up to 10 mg/L total) and a high proportion of AZI-resistant bacteria (> 80%) (Bielen et al., 2017). We also reported high concentrations of macrolides in effluent-receiving Sava river downstream from industrial discharge (up to 30 µg/L total). Furthermore, functional metagenomic analysis of polluted effluents and Sava river sediments identified mostly known (*msr, mph, mef*) and potentially novel (*erm*) macrolide-resistance genes, which were frequently organized in gene clusters and flanked by IS elements (González-Plaza et al., 2018).

Along with its impact on the environmental resistome, antibiotic pollution can affect the microbiome of aquatic ecosystems, by altering its diversity and/or functional properties, as has been demonstrated for different antibiotic classes including macrolides (Grenni et al., 2018).

The present study aimed to comprehensively explore the impact of the discharge of AZI-production effluents on the abundance of macrolide-resistance genes and class 1 integrons, as well as on the composition of bacterial communities in sediments along the receiving Sava river. We then correlated bacterial community with the macrolide-resistance genes and additionally, measured physicochemical properties of sediments, including macrolides, heavy metals and nutrients, to assess environmental factors contributing to community shifts in space and time.

2. Materials and methods

2.1. Study area and sampling

This study was carried out in a section of the Sava River situated in the northwest Croatia. This stretch has been selected due to pollution from an azithromycin-manufacturing industry, which is located approximately 25 km northwest of the city of Zagreb. The final effluent from this industry (after treatment in industry's membrane bioreactor) is discharged, together with effluent from baker's yeast production, into the Sava river near the city of Zaprešić, which makes this location a hot spot for dissemination of antibiotics and ARB/ARGs into the environment (Bielen et al., 2017; González-Plaza et al., 2018). Therefore, we chose to sample surface sediments (0–10 cm) from six locations along the river during winter (February; high-flow, average 1194 m^3/h) and summer (August; low-flow, average 421 m^3/h) of 2016. The sampling sites were 7.5 km upstream of the discharge (UP7500), immediately below the discharge point (DW0) and then progressively downstream at 300 m (just prior to where small river Krapina, which receives effluents from wastewater treatment plant of the city of Zaprešić, enters the Sava river; DW300), 700 m (DW700), 4.5 km (DW4500) and 11 km (DW11000) below the discharge point. Sampling site UP7500 was chosen to represent reference site without any known anthropogenic antibiotic pollution.

From each sampling site, four replicates (about 250 g each) were sampled with a plastic core tube and within approximately 2 square meters at each site. In addition, four effluent samples (about 5 L each) were collected as grab samples at the discharge outflow located at the bank of the Sava river. All samples were stored on ice and transported to the laboratory for immediate processing. Aliquots of effluent (50–100 mL) were vacuum-filtered through a 0.22 µm pore-size membrane (GE Healthcare Life Sciences) to collect bacterial cells; filters were stored at −80 °C until DNA extraction. Subsamples from each of the four replicate sediment samples (2 g) were stored at −80 °C for subsequent DNA extractions. The remaining of the subsamples were composited (10 g of each subsample) and air-dried at ambient temperature for subsequent physicochemical analyses.

2.2. Sediment physicochemical properties

The water content of the sediment samples was determined by the difference in mass after oven drying at 105 °C to constant mass. Dried samples were coarse ground to < 2 mm, and the physicochemical properties, including pH, concentration of total organic carbon (TOC), total carbon (TC), total nitrogen (TN), total phosphorus (TP), nitrite, nitrate and ammonia nitrogen were determined using internationally validated methods (ISO standards) as shown in Table S1. Size fractions were measured with a Laser Coulter LS 13320 diffractometer (Beckman Coulter, USA).

2.3. Chemical analyses of antibiotics and metals

The target macrolides were extracted from the 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; Senta et al., 2013). The heavy metals, including Cr, Co, Ni, Cu, Zn, As, Cd and Pb were determined by inductively coupled plasma mass spectrometry (ICP-MS) following the methodology described previously (Dautović et al., 2014).

2.4. DNA extraction and quantitative PCR of macrolide-resistance genes and class 1 integrons

Total DNA was extracted directly from sediment samples and from frozen filters using the Power Soil DNA isolation kit (MoBio, USA)

according to the manufacturer's recommendations. Non-template sample (DNA-free water) was included as a negative control during the whole workflow. The extraction yield and quality of the DNA were assessed by spectrophotometry (BioSpec Nano, Shimadzu, Japan); DNA quantity was measured by fluorometry (Qubit Fluorometer 3.0, Thermo Fisher Scientific, USA). All extractions were stored at −20 °C until used. Quantitative PCR (qPCR) was used to quantify five macrolide-resistance genes (*mef*C, *mph*G, *mph*E, *msr*E, and *erm*B) that were previously identified in this study area by functional metagenomics (González-Plaza et al., 2018) and the class 1 integron-integrase gene (*int*I1). In addition, the 16S rRNA gene copy numbers (*rrn*) were determined to assess the total bacterial load and to calculate the relative abundance of the resistance genes targeted in the collected samples. All qPCR assays were performed on the ABI 7300 Real-time PCR System (Applied Biosystems, USA) using SYBR Green detection chemistry. The primers used for the ARGs, *rrn* and *int*I1 gene quantification are listed in Table S2. Each reaction was carried out in a total volume of 20 µL containing 10 µL Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), 1 µM of each primer, and 2 ng of DNA template. For all ARGs, except *erm*B, the thermal cycling conditions were as follows: 95 °C for 15 min, 30 cycles at 95 °C for 15 s, annealing temperature (T_m) specific for each gene and primer pair (Table S2) for 30 s, and 72 °C for 30 s. For quantification of *erm*B, thermal cycling conditions were settled in accordance to Chen et al. (2007), for *rrn* according to López-Gutiérrez et al. (2004), and for *int*I1 in accordance with Barraud et al. (2010). Standard curves were constructed as previously described (López-Gutiérrez et al., 2004). Briefly, the target gene fragments were amplified from the positive metagenomic clone (González-Plaza et al., 2018), separated by 1.5% agarose gel electrophoresis and recovered with a QIAquick Gel Extraction Kit (QIAGEN, Germany), and then cloned into pGEM®-T Easy Vector (Promega, France) according to the manufacturer's recommendation. The recombinant plasmids were transformed into JM109 competent cells (Promega, France). Positive clones were checked with PCR and confirmed by Sanger sequencing (Macrogen, Netherlands). The plasmids extracted from them were used to prepare standards for qPCR assays. The efficiency and sensitivity of each qPCR assay (Table S2) was determined by generating a standard curve using 10-fold serial dilutions of the plasmid DNA (10^2–10^8). All detections in the qPCR assay were conducted with four technical replicates, and non-template control were likewise included in each assay. Possible qPCR inhibition was assessed by conducting an inhibition test using samples diluted to 1 ng/µL and 0.01 ng/µL, as previously described (Petric et al., 2011), and no inhibition was observed. Quantitative PCR data were expressed as the ratio of ARG/*int*I1 gene copy number per *rrn* copy number to evaluate the relative proportion of target gene in the bacterial community of each sample.

2.5. Amplicon sequencing and data processing

To profile bacterial communities, the V1-V2 region of 16S rRNA genes was amplified with universal primers 27F and 357R (Klindworth et al., 2013). Amplicon library was prepared as described previously (Gschwendtner et al., 2016) with some modifications. All samples was purified with Nucleospin Gel and PCR Clean-up kit (MACHEREY NAGEL GmbH & Co., Germany), and fragment size and concentration were validated using a FragmentAnalyzer device on a Standard Sensitivity NGS Fragment Analysis kit (Advanced Analytical, USA). Samples were sequenced on a MiSeq instrument (Illumina, United Kingdom, Chesterford) using the MiSeq Reagent Kit v3 for 600 cycles. Sequencing adapters were removed using AdapterRemoval (Schubert et al., 2016). Reads were analyzed using QIIME 2 v2018.2.0 (https://qiime2.org) and denoised using the DADA2 (Callahan et al., 2016) plugin. Resulting amplicon sequencing variants (ASVs) were compared to the 99% identical clustered SILVA database v132 (Quast et al., 2013) using a naive Bayes classifier trained on the amplified region. Alpha diversity was described for each sample using the metrics of observed species (i.e. ASVs), and rarefaction curves were generated to compare the level of bacterial ASVs diversity.

Non-metric multidimensional scaling (NMDS) analysis was performed to evaluate the overall composition of the bacterial community among different sites and seasons based on Bray-Curtis distance by using Canoco software (v5.1). A heat map visualization of the relative abundance of taxa (number of ASVs per number of sequences expressed in percentage) significantly increased at DW0 site compared to UP7500 reference site was performed with the heatmap.2 function of the 'gplots' package (Warnes et al., 2016).

2.6. Statistical analyses

qPCR data (ARG/*int*I1 copy number per *rrn* copy number) were log10 transformed and subjected to a Shapiro-Wilk test to evaluate its normal distribution. This was performed in R studio v1.1.383 with the 'fitdistrplus' and 'stats' packages. These data were further compared using the one-way analysis of variance (ANOVA) or Kruskal-Wallis test (if data were not normally distributed) and Tukey's multiple comparison test. Paired *t*-test was performed to compare the relative abundance of macrolide-resistance genes at each site between two seasons. Data were analyzed using GraphPad Prism V6.01. The package DESeq2 (Love et al., 2014; Jonsson et al., 2016) version 1.22.1 was applied to analyze the differences in relative abundance of bacterial community at phylum and at genus level between each DW site and upstream (UP7500) site. All statistical tests were considered significant at $p < 0.05$. Redundancy analysis (RDA) and Mantel test based on Bray Curtis distance were performed to determine the correlation between bacterial community and physicochemical parameters as well as between bacterial community and macrolide-resistance genes by using Canoco software (v5.1). Shannon diversity, Mantel test and Adonis test were performed using R studio software v1.1.383 with 'vegan' package (Oksanen et al., 2018).

3. Results

3.1. River sediment characteristics

Physicochemical characteristics of river sediments including particle grain-size and nutrient content are summarized in Table S3. The sediment samples from all sampling sites were characterized by alkaline pH values (7.7–9.1) and were generally of sandy-silt texture (Wenthworth, 1922), with sand and silt content ranging from 47 to 85% and 12 to 47%, respectively. In general, discharge of industrial effluents influenced most measured parameters at sites in close proximity to the discharge point (DW0-DW700). The discharge site (DW0) was unique among all sites having the highest concentrations of total carbon (up to 9.06%), total nitrogen (up to 0.26%), nitrite (up to 10 mg/kg) and ammonium (up to 296 mg/kg) as well as conductivity (up to 951 mS).

The concentrations of macrolides (azithromycin, AZI, and erythromycin-H_20, ERY-H_2O) were measured in the sediments to estimate the presence of selection pressure from antibiotics (Table 1). Both macrolides could not be detected in sediments sampled upstream (UP7500 site), except AZI in very low level during winter. In contrast, very high levels of AZI were detected at DW0 site during both seasons (9 and 23 mg/kg). These concentrations decreased with distance further downstream but were still high (> 1 mg/kg) at sites located within 700 m of the effluent discharge (DW300 and DW700). The concentrations of ERY-H_2O were much lower compared to AZI, being the highest at DW0 (approximately 1 mg/kg).

Heavy metals (As, Cd, Cr, Co, Cu, Pb, Ni and Zn) were found in sediments in a wide range of concentrations (Table 1). In general, most of the measured metals exhibited slightly higher concentrations at DW0 and downstream (DW) sites compared to the upstream site during both seasons. Exceptions were Cu, which increased during both seasons about 5 times at DW0 (48 mg/kg, two-season average) compared to

Table 1
Quantification of macrolides and heavy metals in sediments from different sites along the Sava river in winter and summer sampling campaigns.

			Sampling sites					
Macrolide compound (μg/kg of dry sediment)		Season	UP7500	DW0	DW300	DW700	DW4500	DW11000
Azithromycin (AZI)		Winter	4.60	9307	2686	1240	174	251
		Summer	< LOD	23,685	3598	1271	940	203
Erythromycin-H_2O (ERY-H_2O)		Winter	< LOD	869	24	13	6.60	3.30
		Summer	< LOD	939	71	11	6.80	6.00
Metal (mg/kg of dry sediment)	TEC*/MCC**							
Arsenic (As)	9.79/–	Winter	5.81	7.31	6.38	6.44	8.30	5.57
		Summer	5.11	5.00	5.40	9.48	7.23	5.44
Cadmium (Cd)	0.99/1.0	Winter	0.24	0.27	0.28	0.33	0.16	0.23
		Summer	0.27	0.37	0.24	0.46	0.26	0.25
Chromium (Cr)	43.4/–	Winter	37	34	*57*	41	*76*	26
		Summer	26	*74*	29	*58*	41	28
Cobalt (Co)	–/–	Winter	5.17	4.03	5.52	6.02	11	4.30
		Summer	4.42	4.92	4.29	12	6.15	5.03
Copper (Cu)	31.6/11.5	Winter	11	***41***	**18**	**16**	**28**	11
		Summer	9.42	***55***	**17**	**25**	**13**	**13**
Lead (Pb)	35.8/–	Winter	26	26	25	27	35	19
		Summer	15	27	18	*43*	21	18
Nickel (Ni)	22.7/–	Winter	17	*29*	18	20	*47*	13
		Summer	13	20	15	*44*	20	17
Zinc (Zn)	121/42.5	Winter	**79**	***148***	**76**	**83**	***150***	**49**
		Summer	***357***	***165***	**68**	**104**	**72**	**66**

Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream. LOD, limit of detection; the values in italic represent the concentration of the heavy metals above the threshold effect concentration (TEC*). The values in bold represent the concentration of heavy metals above the minimum co-selective concentration (MCC**), above which selection of antibiotic resistance is expected to occur (Seiler and Berendonk, 2012).

UP7500 (10 mg/kg, two-season average) and Zn, which increased about 2 times at DW0 (148 mg/kg) compared to UP7500 (80 mg/kg) only during winter. A surprisingly high concentration of Zn was measured in sediments from UP7500 site in summer (357 mg/kg), being even higher than that measured at DW0 site (165 mg/kg; Table 1), but the source of this pollution is unknown. Notably, concentrations of both Cu and Zn in the sediment at all DW sites exceeded in most cases concentrations needed to co-select for metal resistance and antibiotic resistance, i.e. the minimum co-selective concentrations (MCC) (Seiler and Berendonk, 2012). Additionally, levels of both Cu and Zn in the sediment at DW0 were higher than concentrations expected to induce adverse effects on sediment dwelling organisms, i.e. threshold effect concentrations (TEC) (MacDonald et al., 2000).

3.2. Elevated abundance of macrolide-resistance genes and class 1 integrons in sediments along the Sava River

We assessed the relative abundances of five macrolide-resistance genes (*mph*G, *mph*E, *msr*E, *mef*C and *erm*B) and an integrase gene (*int*I1) of class 1 integrons in effluents and sediments of the receiving river collected in winter and summer sampling campaigns using qPCR (Fig. 1). All values were normalized to 16S rRNA gene (*rrn*) abundances to minimize the variance in background bacterial abundances and to compare our data with ARGs quantitative data from other studies.

Among macrolide-resistance genes in effluents, the most abundant resistance genes in both seasons were *msr*E (up to 5.6×10^{-2} copies/*rrn* copies), *mph*G (up to 2.5×10^{-2} copies/*rrn* copies) and *erm*B (up to 1.1×10^{-2} copies/*rrn* copies), while the abundances of *mef*C and *mph*E were in most cases 10-times lower (approximately 10^{-3} copies/*rrn* copies in winter and 10^{-4} copies/*rrn* copies in summer) (Fig. 1). The relative abundances of all target ARGs, except *erm*B, were significantly higher in winter than in summer effluents ($p < 0.05$; paired *t*-test). Interestingly, *int*I1 gene was even more abundant than ARGs in effluents, with an average value of the two seasons of 6.6×10^{-1} copies/*rrn* copies (Fig. 1).

In sediment samples from UP7500 site, only *mph*G, *msr*E and *erm*B genes were detected at relative abundances of about 10^{-4}–10^{-5} gene copies/*rrn* copies, with *msr*E abundance being the highest (Fig. 1). However, in sediments from DW0 and DW sites, all five resistance genes targeted were detected at significantly higher relative abundances compared with UP7500 site ($p < 0.05$; ANOVA). Highest relative abundances occurred at DW0 site (up to 10^{-1} to 10^{-2} gene copies/*rrn* copies), while at DW sites (DW300 - DW11000), the relative abundance of up to 10^{-3} ARG copies/*rrn* copies was determined. In general, no significant difference was found between relative abundances of ARGs from winter and summer samples from all sites ($p > 0.05$; paired *t*-test).

Compared to ARGs, higher relative abundances of *int*I1 gene were observed in the samples from UP7500 site, with 8.6×10^{-3} copies/*rrn* copies in winter samples and 2.1×10^{-1} copies/*rrn* copies in summer samples indicating higher background levels of *int*I1 gene. The relative abundance of this gene was significantly increased at all DW sites ($p < 0.05$; ANOVA), except DW700 during the winter (Fig. 1). Values were in the range of 10^{-1} gene copies/*rrn* copies at locations DW0 and DW300, and about 10 times lower at other DW sites. The level of *int*I1 in the UP7500 sediment was considerably higher during summer compared to winter (Fig. 1).

3.3. Effects of effluent discharge on bacterial communities

A total of 6,140,241 sequences of 16S rRNA genes amplified from a total of 50 effluent and sediment samples were analyzed. Of that, 6,123,931 sequences were high-quality reads after adapter removal, which corresponded to a range of 70,509 to 207,244 sequences per sample. These sequences were assigned to 26,147 ASVs at 99% similarity level. The number of sequences obtained for the non-template control ranged from 71 to 64,287. Rarefaction curves indicated that the number of sequences was sufficient to cover the vast majority of species in the bacterial community within each analyzed sample (Fig. S1).

Shannon-Wiener diversity index indicated that effluent discharge did not significantly ($p > 0.05$, Kruskal-Wallis) affect the number of taxa in receiving sediments (species richness) during both seasons,

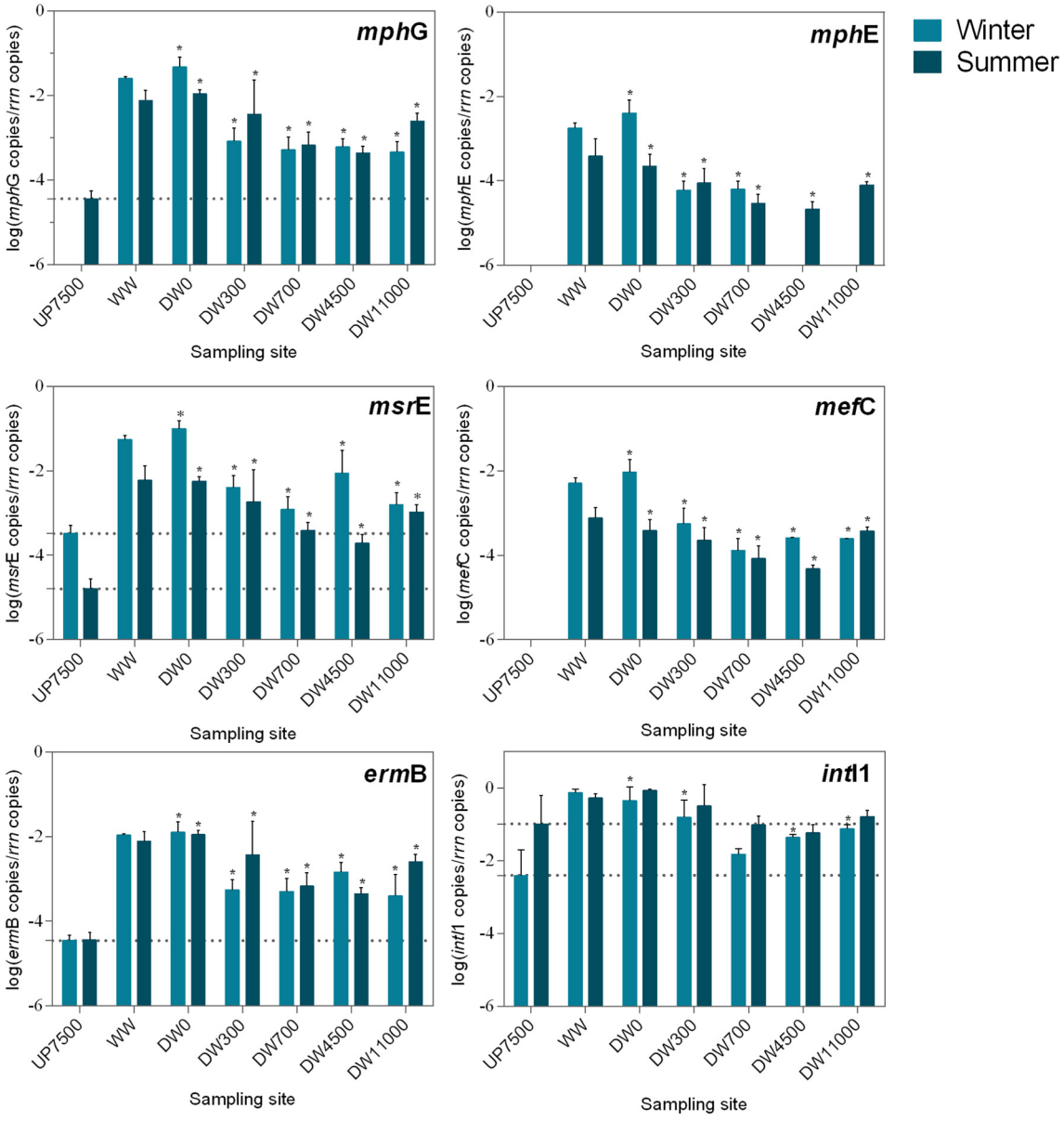

Fig. 1. Relative abundance of macrolide-resistance genes (*mph*G, *mph*E, *msr*E, *mef*C, *erm*B) and class 1 integron-integrase gene (*int*I1) in azithromycin-manufacturing effluents (WW) and sediments of the receiving Sava river from different sites. Each value is the mean ± SD of four replicates. Dotted lines indicate the relative abundances of each gene in background sediment. Asterisks represent a significant difference ($p < 0.05$; ANOVA) between each DW site and reference UP7500 site. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream; WW, effluent.

although sediments from the discharge site tended to have the lowest diversity among all samples (Fig. S2). In addition, the NMDS plots based on Bray Curtis similarity showed a clearly distinct bacterial community composition between sediments from DW0 and the other (UP and DW) sites (Fig. 2; adonis: $R^2 = 0.8675$, $p < 0.05$). Furthermore, bacterial communities varied between the sampling time points at DW0 and the other (UP and DW) sites (Fig. 2; adonis: $R^2 = 0.8772$, $p < 0.05$), implying a seasonal effect on the sediment community composition.

Proteobacteria were dominating in all samples, followed by *Actinobacteria* and *Acidobacteria* in winter upstream and downstream sediment samples and by *Acidobacteria* and *Bacteroidetes* in summer upstream and downstream sediment samples (Fig. S3). In contrast, in effluents and sediments at the discharge site *Bacteroidetes, Epsilonbacteraeota* and *Firmicutes* were, besides *Proteobacteria*, the most abundant phyla independent from sampling season. To pinpoint those phyla which significantly differed between UP7500 and the other sites, DESEq2 analysis was applied ($p < 0.05$). As evident from Fig. 3, effluent discharge significantly decreased relative abundance of a number of phyla at DW0, but not at downstream sites. Compared to UP7500, the highest decrease in relative abundance at DW0 was observed for *Actinobacteria* (23.65% winter, 7.85% summer), *α-Proteobacteria* (11.2% winter, 6.5% summer), *Acidobacteria* (9.21% winter, 7.26% summer), and *Cyanobacteria* (6% summer), whereas for the other three affected phyla (*Chloroflexi, Gemmatimonadetes* and *Nitrospirae*), the decrease in relative abundance was ≤3% for both seasons. In contrast, several phyla showed a significantly higher relative abundance at DW0 compared to UP7500 site during both seasons, including *Epsilonbacteraeota* (11% winter, 5% summer), *Bacteroidetes* (15% winter, 21% summer) and *Firmicutes* (12% winter, 14% summer). Other phyla, such as *Spirochaetae* and *Synergistetes* were detected in significantly higher relative abundance at DW0 in both seasons, but their actual

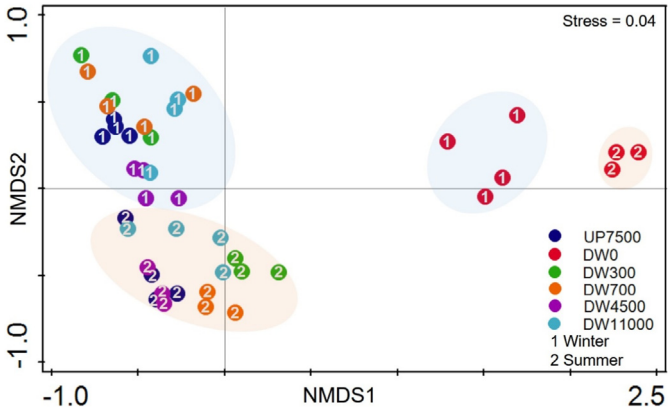

Fig. 2. Spatial and seasonal changes in sediment community composition across three to four replicates of each sampling site along the river. The replicate samples obtained from the same sampling site were marked with the same color, and from the same season with the same number. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream.

relative abundances were, at most, 3% in winter and 4% in summer samples. In addition to this, γ-*Proteobacteria* (12.4%) and *δ-Proteobacteria* (4%) was significantly higher in relative abundance at DW0 only during winter, whereas *Cloacimonetes* (4%) were higher only in samples taken in summer. As *Proteobacteria, Bacteroidetes, Epsilonbacteraeota* and *Firmicutes* were the most abundant phyla in both, effluents and sediments at the discharge site, we further investigated which genera within these phyla were significantly higher at DW0 compared to UP7500. We postulated that they may have represented bacterial taxa that acquired macrolide-resistance genes or are intrinsically resistant, and therefore able to survive and proliferate despite the strong selective pressure from macrolides (and potentially other toxic chemicals). Within *Bacteroidetes*, genera including *Bacteroides, Paludibacter* and vadinBC27 wastewater-sludge group that originated from effluent were significantly higher in relative abundance in both winter- and summer sediment samples; additionally, *Macellibacteroidetes* was more abundant only in winter sediment (Fig. 4 and Tables S4 and S5). Within the *Firmicutes,* the genus *Trichococcus,* that was found in both effluent and background sediment, dominated at DW0 site during both seasons (Fig. 4 and Tables S4 and S5). In addition, ASVs linked to *Erysipelothrix* and *Fusibacter,* which were more abundant in effluent than in background sediment, as well as *Anaerovorax* and *Christensenellacae* R-7 group, specific to effluent, were enriched in winter sediment. However, ASVs which were closely related to *Enterococcus*, *Anaerovorax* and uncultured genera of the order *Selenomonadales,* which originated from effluent, were significantly enriched in sediment samples from the summer sampling (Tables S4 and S5; Fig. 4). Among *Proteobacteria* and *Epsilonbacteraeota*, populations that were enriched at DW0 compared to UP7500 differed between winter and summer sediment samples. The genus *Thauera* was the most abundant in sediment from DW0 site in both seasons but was present at low abundance in the UP7500 sediment (0.01%) and at relatively high abundance (approximately 1%) in effluents (Fig. 4 and Tables S4 and S5). In addition, genera found to be abundant in winter effluent (> 0.5%; e.g. *Commamonas, Variovax)* (Fig. 4a; Table S4) were also highly abundant in winter DW0 sample (Fig. 4a; Table S4). Some genera such as *Rhizobacter, Thiobacillus* and *Methylotenera* that were higher in relative abundance in DW0 compared to the UP7500 sediment in winter, originated from UP7500 sediment, whereas the other genera such as *Gemmobacter, Paracoccus, Propionivibrio, Desulfobulbus, Sulfuricurvum, Arcobacter, Pseudomonas* and *Acinetobacter* were specific to effluent or more abundant in effluent compared to UP7500 sediment. Further, genera such as *Falsirhodobacter, Desulfobacter,* and *Arcobacter,*

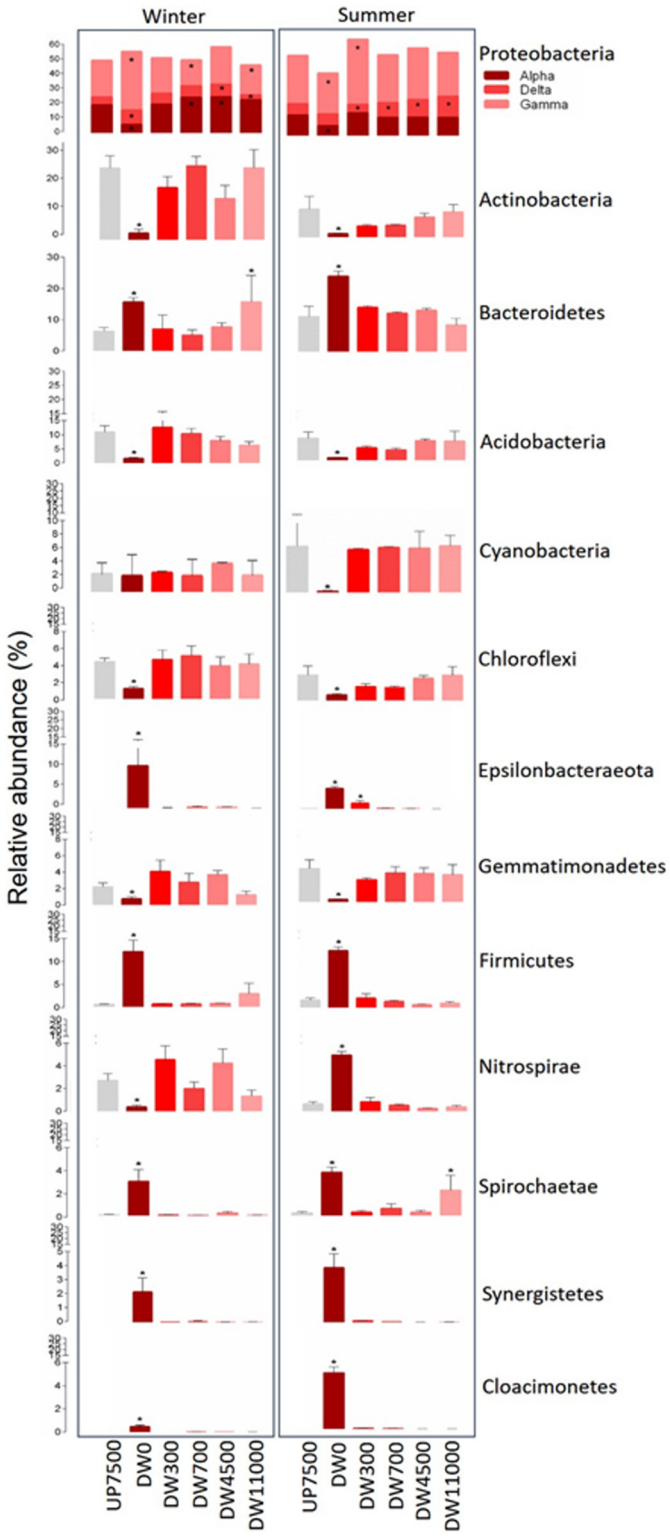

Fig. 3. Phylum-level changes in community composition in sediments from different sites along the Sava river during winter and summer. Relative abundances of phylotype reads are shown, identified to their closest match in the SILVA Database. Asterisks represent a significant difference ($p < 0.05$; DESeq2) between reference UP7500 and each DW site. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream.

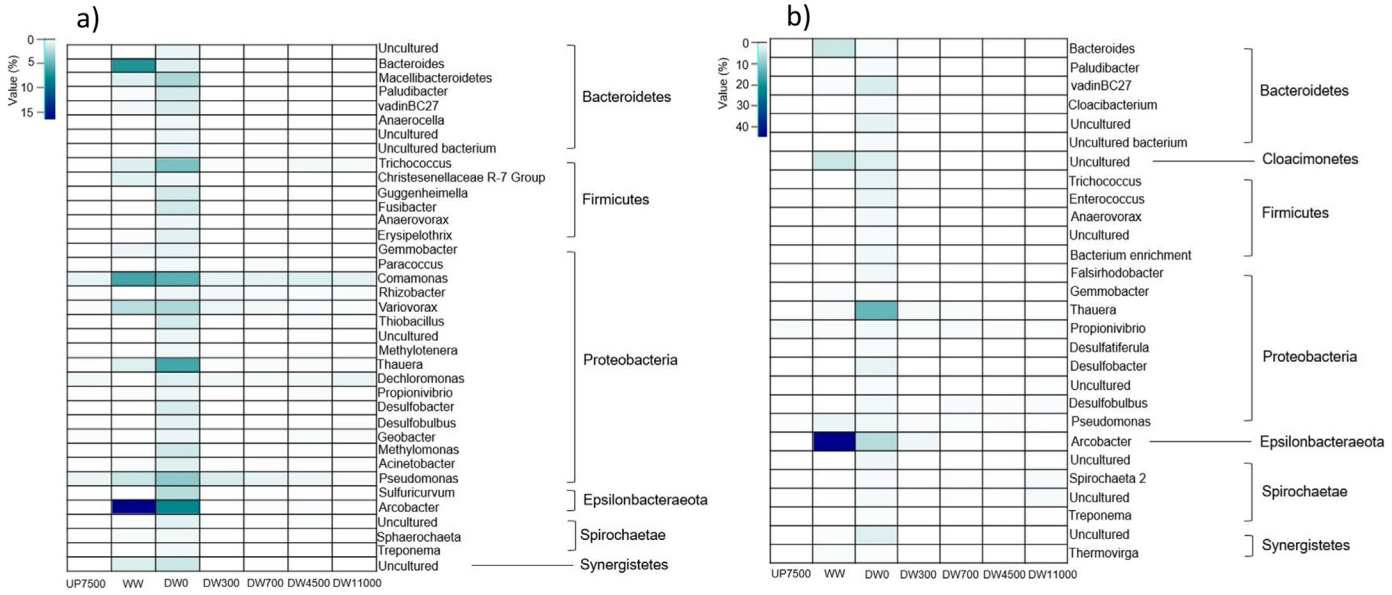

Fig. 4. Heatmaps showing changes in the relative abundance of taxa that were significantly increased at DW0 site compared to UP7500 site during a) winter and b) summer season. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream; WW, effluent.

that were enriched in DW0 during summer originated only from effluent (Fig. 4b; Table S5).

Overall, despite seasonal differences in sediment community composition at DW0 site, the abundance of nearly all taxa higher in relative abundance at this site dramatically decreased at downstream sites, without significant differences in abundance even at site DW300 compared with UP7500 site (Fig. 4). An exception is an effluent-associated *Arcobacter,* which remained significantly enriched at DW300, in addition to DW0, during summer.

3.4. Roles of environmental factors for bacterial community shifts and alteration of macrolide-resistance genes

We performed redundancy analysis (RDA) to study the relationship of sediment physicochemical parameters and bacterial community composition at the genus level during winter and summer (Fig. 5). In line with the NMDS plot (Fig. 2), RDA analysis confirmed shifts in bacterial community structure at DW0 site, which was significantly positively correlated with macrolides (AZI and ERY-H_2O), Cu, and nutrients (TC, TN, NH_4^+ and NO_2^-) during both seasons (Mantel test, $p < 0.05$), but negatively correlated with pH (Mantel test, $p < 0.05$). However, bacterial communities from winter DW0 samples were significantly correlated with Zn and NO_3^- (Fig. 5a), whereas communities from summer DW0 samples significantly correlated with TOC, TP, Cr and Cd (Fig. 5c). Surprisingly, Mantel test revealed no significant correlation between these bacterial communities and temperature (not shown). Therefore, seasonal changes of above-mentioned parameters, in addition to changes in bacterial community composition of effluents (Fig. 4), could be responsible for sediment community shifts between winter and summer.

RDA of the bacterial communities and macrolide-resistance genes showed that *mph, mef, msr* and *erm* genes were significantly correlated to community composition at DW0 site during both seasons (Mantel test, $p < 0.05$; Fig. 5b and d). Among the identified genera, Gram-positive *Trichococcus* and Gram-negative *Thauera* and *Arcobacter* showed a significant positive correlation with analyzed resistance genes at DW0 site during both seasons. In addition, genera such as *Macellibacteroides* (Gram-positive) and *Sulfuricurvum* and *Commanomas* (Gram-negative) positively correlated with macrolide-resistance genes at DW0 during winter (Fig. 5b), whereas vadinBC27 wastewater-sludge group and uncultured populations from *Bacteroidetes, Cloacimonetes* and *Synergistetes* exhibited a significant positive correlation with macrolide-resistance genes during summer (Fig. 5d).

4. Discussion

This study provided comprehensive insights into the effects of the discharge of treated effluents from AZI-manufacturing on macrolide-resistance genes and bacterial communities of the receiving Sava river sediment. We recently showed that effluents from this industry contained high numbers of AZI-resistant bacteria and macrolide antibiotics at concentrations clearly selective for antibiotic resistance (up to few mg/L; Bielen et al., 2017). Accordingly, direct discharge of these effluents could significantly affect the bacterial communities and the macrolide-resistance genes of the receiving river, which might pose a risk for human and environmental health. Although sediments act as a drain for pollutants, they can also act as a source of pollutants under certain environmental conditions, especially during high water-flow events (Herrero et al., 2018). We, therefore, investigated river sediments impacted by industrial effluents during both high-flow (winter) and low-flow (summer) sampling conditions.

4.1. Chemical pollution of river sediments by industrial effluent discharge

We detected high levels of macrolide antibiotics, especially AZI, in the effluent-impacted sediments, with the highest levels at the discharge site (up to 23 mg/kg) and a sharp decrease to about 1 mg/kg at the site located 700 m downstream. This indicated contribution of industrial waste to macrolide pollution of sediments from the Sava river, in addition to pollution of river surface water (Bielen et al., 2017). Although higher macrolide concentrations were previously found in winter than in summer effluent samples (Bielen et al., 2017), the reverse situation was observed for sediment samples. Lower concentrations of macrolides detected in sediment samples taken in winter compared to summer could be due to the higher flow rate of the river and increased sediment transport during winter or lower amount of effluent discharge during winter compared to summer. Although it is difficult to estimate the degree of bioavailability of the detected macrolides in the sediment, it has been shown using data generated for tylosin that macrolides retain their antimicrobial activity even when

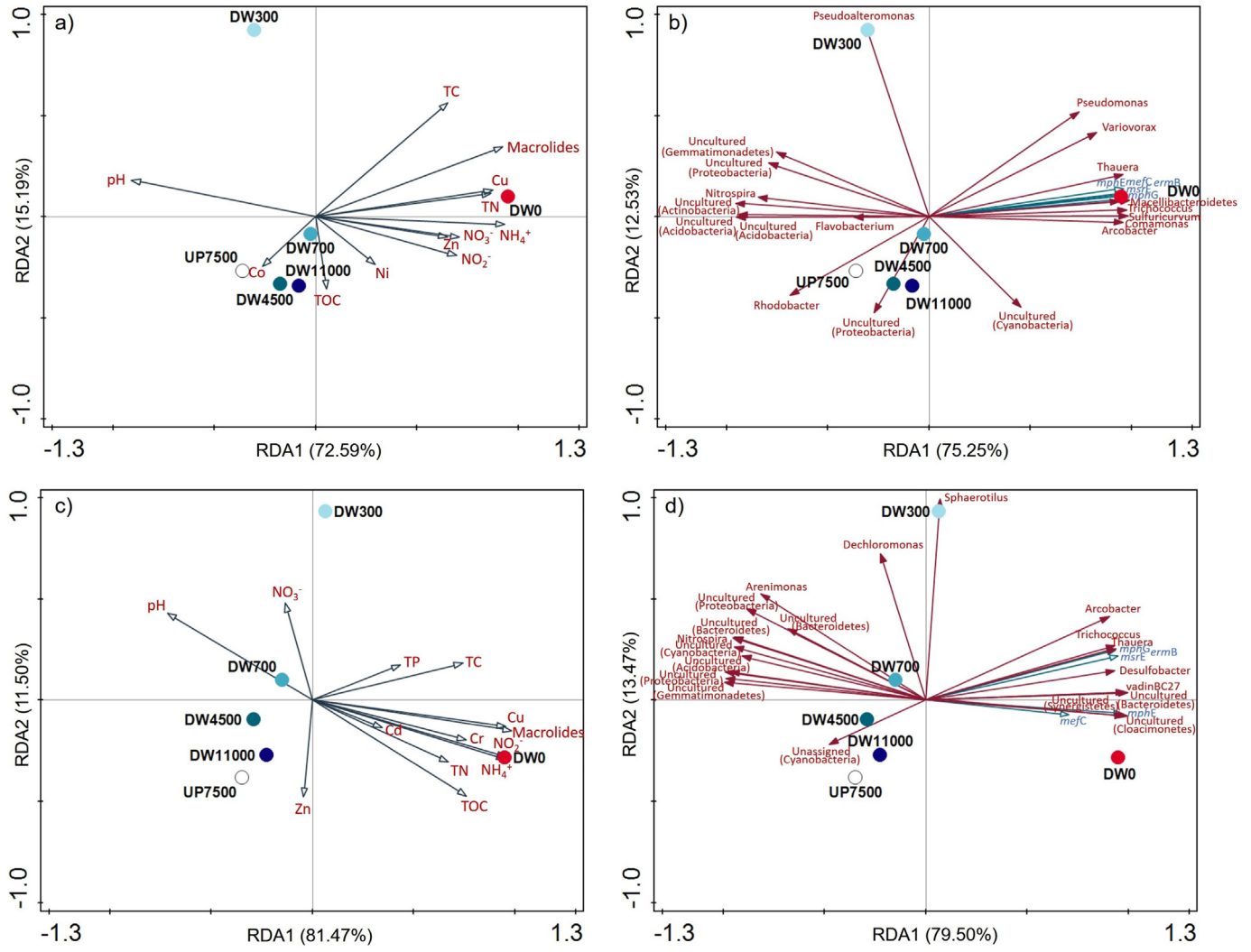

Fig. 5. Redundancy analysis (RDA) of the quantitative correlation between bacterial communities at genus level (> 2% in any sample) and physicochemical sediment parameters during a) winter and c) summer. RDA for the relationship between bacterial communities and relative abundance of macrolide-resistance genes detected in Sava river sediments during b) winter and d) summer season. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream.

tightly adsorbed by clay particles (Chander et al., 2005). Therefore, measured macrolide levels, at least at the most polluted DW0 site, may exert a strong selective pressure to select for macrolide resistant bacteria. Macrolide levels detected in river sediments in this study were lower than levels of fluoroquinolones discharged from bulk drug production in the Indian river sediments from Patancheru area (up to 54 mg/kg), but higher than fluoroquinolone levels observed in the Musi river sediments in India (up to 3 mg/kg), which were also exposed to effluents from drug manufacturing (Gothwal and Shashidhar, 2017). In addition to antibiotics, direct discharge of analyzed effluents led to the accumulation of heavy metals, especially Cu and Zn, in exposed sediments, suggesting metal pollution from incoming industrial waste. Based on the levels of Cu and Zn detected in sediments, which were above their MCCs (Seiler and Berendonk, 2012), they might contribute to co-selection of metal resistance and antibiotic resistance. There are numerous studies where metals have been reported to promote antibiotic resistance via co-occurrences of metal and antibiotic resistance genes (Di Cesare et al., 2016b; Guo et al., 2018; Pal et al., 2015; Song et al., 2017). For example, resistance to Cu in bacteria has already been linked with resistance to macrolides and to other antibiotic classes (Amachawadi et al., 2011; Hasman and Aarestrup, 2002). Therefore, the resulting selection pressure from Cu and Zn may have contributed to the development of macrolide resistance via co-selection. In addition to this, the concentration of Cu, Zn, Ni, and Cr were above their TEC values suggesting that these metals could have toxic effects on sediment-dwelling organisms. Together with antibiotics and metals, significant amounts of organic material, phosphorous and nitrogen compounds were released through effluent discharge into the Sava river (Bielen et al., 2017), elevating concentrations of C, N and P compounds in receiving sediments, especially at DW0 site, and modifying the structure of bacterial communities, promoting horizontal gene transfer and the abundance of ARGs.

4.2. Enrichment of macrolide-resistance genes and class 1 integrons in effluent-receiving sediments

Among the five targeted macrolide-resistance genes, three genes encoding macrolide phosphotransferase (*mph*G), ribosome protection protein (*msr*E) and ribosomal methylase (*erm*B) were the dominant subtypes detected in the analyzed effluents, and their relative abundance was significantly elevated in effluent-receiving sediments. In addition, the *mef*C gene and the *mph*E gene encoding for efflux and macrolide phosphotransferase, respectively, were found somewhat less prevalent in effluents compared to *msr*E, *mph*G and *erm*B genes, but

were also significantly more abundant in exposed sediments. Therefore, the discharge of industrial effluents contributed to increased levels of macrolide-resistance genes in the sediments of the receiving river. All resistance genes targeted were found to be persistent in sediments until 11 km downstream. Surprisingly, we found no significant effect of the season on the distribution of target resistance genes in sediments, despite higher river water flow in winter compared to summer. It has been shown that the relative abundance of ARGs in severely polluted areas can reach values of 10^{-2}–10^{-1} copies/*rrn* copies (Gao et al., 2018), which is comparable to the relative abundances observed in effluents and river sediments at the discharge site in this study. However, although the average relative abundance of the most prevalent resistance genes (*mph*G, *msr*E and *erm*B) was lower in downstream sediments (up to 10^{-3} gene copies/*rrn*), the complete downstream section of the Sava river was generally polluted by macrolide-resistance genes. The observed levels at downstream sites were comparable to those reported by Rutgersson et al. (2014), who quantified quinolone-resistance genes, especially *qnr*VC gene, at approximately 10^{-3} gene copies/*rrn* copies in Indian river sediments polluted with mg/L levels of fluoroquinolones. Other studies also showed that highly polluted sites often have $> 10^{-4}$ ARG copies/*rrn* copies (Gao et al., 2018; Graham et al., 2011). Many macrolide-resistance genes including those targeted here have commonly been found on conjugative plasmids that often carry other ARGs (Sugimoto et al., 2017; Zhang et al., 2013). These plasmids can transfer between different bacterial populations and therefore promote the acquisition and spread of multiple resistance genes, contributing to their persistence in the environment.

In addition to target *msr, mph, erm* and *mef* genes, the relative abundance of the *int*I1-associated class 1 integrons was significantly higher in sediments sampled at the discharge and downstream sites during the winter, which indicated contribution of industrial effluent to increased abundance of class 1 integrons. The class 1 integron-integrase gene, *int*I1, was previously proposed as a genetic marker for anthropogenic pollution (Gillings et al., 2015) and its plasmid-localization would facilitate potential for HGT in these sediments. However, investigated sites, especially the highly impacted ones (DW0-DW300) were similar in both seasons regarding *int*I1 gene levels. Thus, the lack of *int*I1 genes at significantly elevated levels above background during summer is due to its increased level at the upstream site.

4.3. Changes in sediment bacterial community in response to pharmaceutical pollution

At the phylum level, the composition of bacterial communities in sediments of Sava river is typical for river sediment, with *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Acidobacteria* among the major groups (Ibekwe et al., 2016; Wang et al., 2018; Xie et al., 2016). The effluent discharge was found to shift community composition in sediments at the discharge site. *Firmicutes, Bacteroidetes* and *Epsilonbacteraeota* were significantly increased in relative abundance and dominated together with *Proteobacteria* at DW0 site and in effluents during both seasons. These results are consistent with the observation of Kristiansson et al. (2011), who demonstrated high abundance of the same three phyla in antibiotic-polluted river sediments in India. Our RDA analysis further suggested that macrolide and Cu concentration together with the concentration of some nutrients (TC, TN, NH_4^+ and NO_2^-) at least partially contributed to this community shift at the discharge site. The number of taxa was significantly increased, including those originated from effluent (*Macellibacteroidetes, Sulfuricurvum* or vadinBC27 group) and those that were found in UP7500 sediment and effluent (*Trichococcus, Thauera, Arcobacter, Pseudomonas, Variovax* and *Comamonas*) (Fig. 5). Therefore, deposition of effluent-associated bacteria together with the proliferation of resident sediment bacteria in nutrient-rich conditions under selection pressure from antibiotics and heavy metals evidently took place at the discharge site during both seasons. However, the proportion of taxa that were significantly increased in abundance at DW0 decreased sharply (proportion < 1%) at the downstream sites. Already at the site 300 m downstream, abundances found were comparable to the upstream UP7500 site. These observations are consistent with NMDS data (Fig. 2) showing the similar structure of bacterial communities in upstream and downstream sediment samples. These findings suggested that populations that were significantly increased in relative abundance at DW0 site were either not transported further downstream or could not survive and establish at downstream sites. Indeed, the majority of introduced effluent-associated bacteria might not be very well adapted to sediment, allowing the resilience of the indigenous sediment bacterial community. Furthermore, seasonal changes in effluent community composition along with changes in the chemical properties of sediments could be responsible for community shifts between winter and summer.

We found that both Gram-positive (*Macellibacteroidetes, Trichococcus)* and Gram-negative genera (*Macellibacteroidetes, Sulfuricurvum, Thauera, Arcobacter, Pseudomonas, Variovorax, Comamonas*) that had a significantly higher relative abundance at the discharge site were positively correlated with the distribution of *mph, msr* and *mef* genes, suggesting that they might at least partially influence the abundance of these genes in sediments. However, we cannot exclude the possibility that some or all of these bacteria are intrinsically resistant to macrolides. Identified genera were previously reported in different wastewater treatment facilities (Cydzik-Kwiatkowska and Zielińska, 2016; Dichosa et al., 2015; Zhang et al., 2017), and in river sediments polluted with effluents from these facilities (Lu and Lu, 2014; Martínez-Santos et al., 2018), antibiotics (Nakayama et al., 2017) or heavy metals (Suhadolnik et al., 2017; Zhao et al., 2014).

Despite the resilience of the bacterial communities in river sediments, the abundances of target macrolide-resistance genes were still maintained at elevated levels at downstream sites. This may be an indication of horizontal transfer of ARGs to new hosts or the persistence of extracellular ARGs or combination of both. Our previous analysis of the regions flanking macrolide-resistance genes targeted here suggested that these genes likely originated from plasmids (González-Plaza et al., 2018). This further suggests that analyzed resistance genes are candidates for dissemination to other bacteria in river sediment. This is also supported by our most recent exogenous isolation of conjugative broad host range plasmids conferring macrolide resistance from sediments downstream of industrial discharge point (unpublished data). Together, these data indicate that the transferable resistome is likely the primary mechanism for persistence of macrolide-resistance genes in downstream sediments.

Finally, this study together with studies on Asian production facilities provides further evidence for the importance of aquatic environments receiving effluents from antibiotic production for the selection and dissemination of antibiotic resistance. Therefore, to mitigate resistance dissemination, improvements in the management of pharmaceutical discharges are urgently needed also in Europe. These improvements should include increasing the efficiency of existing wastewater treatment technologies through implementation of advanced posttreatment methods to lower the concentration of both antibiotics and ARB/ARGs in the treated effluents (Gadipelly et al., 2014; Rizzo et al., 2013). In addition, improved management of industrial discharges also requires defining and implementing emission limits for both antibiotics and ARB/ARGs to reduce their releases into recipient environments.

5. Conclusion

In conclusion, this study revealed that discharge of insufficiently treated effluents from azithromycin production contributed to the chemical and biological pollution of sediments from receiving river, which resulted in a spatial and seasonal shift of sediment bacterial community, and led to the enrichment of resistance genes to clinically important macrolide antibiotics. The latter means more opportunity for

capture of these resistance genes by pathogens, exacerbating the risk of human exposure to resistant pathogens through drinking water, the food chain or recreation activities in a polluted river. To reduce such risks, more efforts should be devoted to better management strategies for keeping down the antibiotic and ARB/ARGs pollution levels from antibiotic production.

Data accessibility

The 16S rRNA gene sequences that support the findings of this study have been deposited in GenBank within the BioProject with the accession code PRJNA508592.

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.12.050.

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