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Abstract

Environmental discharges of very high (mg/L) antibiotic levels from pharmaceutical production contributed to the selection, spread and persistence of antibiotic resistance. However, the effects associated with discharges of less antibiotic-polluted effluents (µg/L) from drug-formulation are still scarce. Here we analyzed formulation effluents and sediments from the receiving creek collected at the discharge site (DW0), upstream (UP) and 3000 m downstream of discharge (DW3000) during winter and summer season. Chemical analyses indicated the largest amounts of trimethoprim (up to 5076 µg/kg) and azithromycin (up to 389 µg/kg) at DW0, but sulfonamides accumulated at DW3000 (total up to 1175 µg/kg). Quantitative PCR revealed significantly increased relative abundance of various antibiotic resistance genes (ARGs) against ß-lactams, macrolides, sulfonamides, trimethoprim and tetracyclines in sediments from DW0, despite relatively high background levels of some ARGs already at the reference site (UP). However, only sulfonamide (sul2) and macrolide ARG subtypes (mphG and msrE) were still elevated at DW3000 compared to UP. Sequencing of 16S rRNA genes revealed pronounced changes in the sediment bacterial community composition from both DW sites compared to UP site, regardless of the season. Numerous taxa with increased relative abundance at DW0 decreased to background levels at DW3000, suggesting die-off or lack of transport of effluent-originating bacteria. In contrast, various taxa that were more abundant in sediments than in effluents increased in relative abundance at DW3000 but not at DW0, possibly due to selection imposed by high sulfonamide levels. Network analysis revealed strong correlation between some clinically relevant ARGs (e.g. blaGES, blaOXA, ermB, tet39, sul2) and taxa with elevated abundance at DW sites, and known to harbour opportunistic pathogens, such as Acinetobacter, Arcobacter, Aeromonas and Shewanella. Our results demonstrate the necessity for improved management of pharmaceutical and rural waste disposal for mitigating the increasing problems with antibiotic resistance.

Keywords	antibiotic manufacturing; sediment; pollution; bacterial community; antibiotic resistance genes

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September 16, 2019

Dear Editor,

My co-authors and I are pleased to submit the research paper entitled "*Effects of industrial effluents containing moderate levels of antibiotic mixtures on the abundance of antibiotic resistance genes and bacterial community composition in exposed creek sediments*" for consideration at Environmental Pollution.

This paper contributes to understanding the potential for antibiotic manufacturing practices to contribute to clinical antibiotic resistance. Despite many claims, there are surprising gaps in our understanding of origins and movement of resistance genes in environments impacted by manufacturing discharges and the potential for exchange between environmental and clinical settings. In this study, we demonstrate that discharges of effluents from drug-formulation industry enrich antibiotic resistance genes and alter sediment bacterial community composition of the receiving creek. These observations have important implications, given the high rates of gene exchange between aquatic bacteria and the potential for human consumption of resistant pathogens through eating/drinking contaminated food/water. Therefore, our results provide important insights into a topic of global urgency for human and environmental health.

We believe that this contribution will interest a broad diversity of microbiologists because it spans environmental, industrial and clinical microbiology, and presents methodology that is highly relevant to the study of all microbial communities.

Thank you in advance for your consideration. We look forward to your response.

Sincerely,

Nikolina Udiković-Kolić

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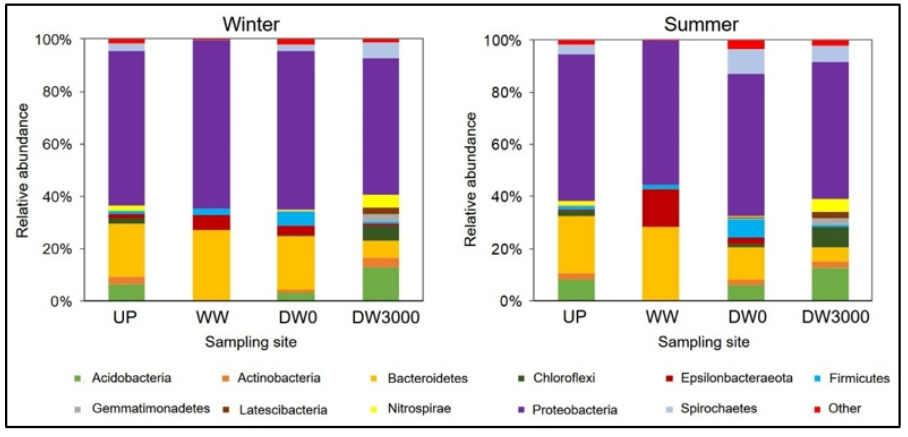
Highlights:

- Antibiotic pollution of creek sediments receiving drug-formulation effluents
- Increased relative abundance of most target ARGs in sediments from discharge site
- Three ARG subtypes had increased relative abundance even 3 km downstream of the emission source
- Spatial shifts of bacterial community composition in exposed sediments
- Associations between increasing ARGs and potential bacterial hosts

spatial shift in sediments bacterial community composition



300m upstream UP

antibiotics

ARGs

sediment DW0

effluent discharge

WW

Antibiotic-formulation industry

antibiotics

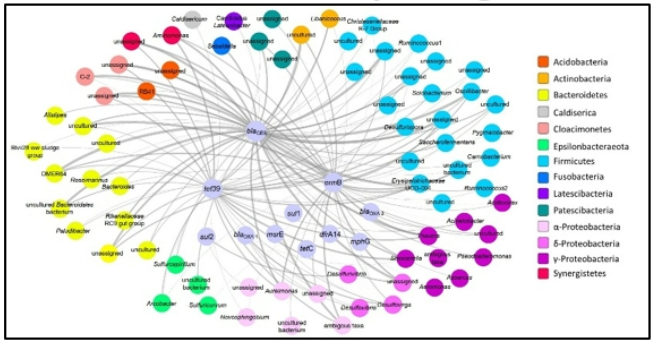
ARGs

sul2, msrE, mphG

sediment DW3000

3km downstream

correlation between clinically relevant ARGs and taxa known to harbor human pathogens



Effects of industrial effluents containing moderate levels of antibiotic mixtures on the abundance of antibiotic resistance genes and bacterial community composition in exposed creek sediments

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ABSTRACT

Environmental discharges of very high (mg/L) antibiotic levels from pharmaceutical production contributed to the selection, spread and persistence of antibiotic resistance. However, the effects associated with discharges of less antibiotic-polluted effluents (µg/L) from drug-formulation are still scarce. Here we analyzed formulation effluents and sediments from the receiving creek collected at the discharge site (DW0), upstream (UP) and 3000 m downstream of discharge (DW3000) during winter and summer season. Chemical analyses indicated the largest amounts of trimethoprim (up to 5076 µg/kg) and azithromycin (up to 389 µg/kg) at DW0, but sulfonamides accumulated at DW3000 (total up to 1175 µg/kg). Quantitative PCR revealed significantly increased relative abundance of various antibiotic resistance genes (ARGs) against ß-lactams, macrolides, sulfonamides, trimethoprim and tetracyclines in sediments from DW0, despite relatively high background levels of some ARGs already at the reference site (UP). However, only sulfonamide (*sul*2) and macrolide ARG subtypes (*mph*G and *msr*E) were still elevated at DW3000 compared to UP. Sequencing of 16S rRNA genes revealed pronounced changes in the sediment bacterial community composition from both DW sites compared to UP site, regardless of the season. Numerous taxa with increased relative abundance at DW0 decreased to background levels at DW3000, suggesting die-off or lack of transport of effluent-originating bacteria. In contrast, various taxa that were more abundant in sediments than in effluents increased in relative abundance at DW3000 but not at DW0, possibly due to selection imposed by high sulfonamide levels. Network analysis revealed strong correlation between some clinically relevant ARGs (e.g. bla_{GES}, bla_{OXA}, *erm*B, *tet*39, *sul*2) and taxa with elevated abundance at DW sites, and known to harbour opportunistic pathogens, such as *Acinetobacter*, *Arcobacter*, *Aeromonas* and *Shewanella*. Our

results demonstrate the necessity for improved management of pharmaceutical and rural waste disposal for mitigating the increasing problems with antibiotic resistance.

Keywords: antibiotic manufacturing; sediment; pollution; bacterial community; antibiotic resistance genes

1. INTRODUCTION

The rise in antibiotic resistance (AR) represents a serious and growing threat for human health worldwide (O'Neill, 2016). Highly similar or even identical antibiotic resistance genes (ARGs) have been found in both environmental and pathogenic bacteria (Poirel et al. 2005; Forsberg et al. 2012; Zhou et al., 2018), emphasizing a potentially shared resistome. Under a selection pressure from antibiotics or from a combination of antibiotics and other co-selective agents (metals, biocides), e.g. caused by discharges from antibiotic production facilities, the environmental resistome becomes enriched with antibiotic-resistant bacteria (ARB) and ARGs they carry (Milaković et al., 2019, Lubert et al., 2017, Šimatović and Udiković-Kolić, 2019). This increase in ARGs was invariably accompanied by the increased occurrences of mobile genetic elements (MGEs) associated with ARG transfer (González-Plaza et al., 2019; Kristiansson et al., 2011; Flach et al., 2015), and a recent study showed that a significantly larger fraction of ARGs are indeed potentially mobilized after antibiotic selective pressure (Sáenz et al., 2019). Consequently, environments polluted by discharges from antibiotic manufacturing have been identified as ´high risk´ environments for AR selection and dissemination into human or animal pathogens. It is, therefore, of urgent concern to investigate such contaminated areas for determining the abundance of AR and identifying the critical control points to reduce its emergence and spread (Šimatović and Udiković-Kolić, 2019).

Large environmental pollution from the antibiotic manufacturing sector was reported to be a problem mostly in Asian countries, such as India, China, Korea and Pakistan, but also, to a lesser extent, in Europe (Larsson et al., 2014; Bielen et al., 2017; Šimatović and Udiković-Kolić, 2019). Very high, mg/L-levels of antibiotics have been detected in effluents from antibiotic production facilities in above-mentioned countries, which led to high antibiotic

pollution as well as the selection, maintenance and spread of AR in the receiving aquatic environment (Flach et al., 2015; González-Plaza et al., 2019; Larsson et al., 2014; Milaković et al., 2019; Šimatović and Udiković-Kolić, 2019). Additionally, the exposure to these effluents introduced various toxic effects in fish and other aquatic organisms as well as pronounced changes in exposed aquatic bacterial communities (Bielen et al., 2017; Milaković et al., 2019; Kristiansson et al., 2011; Carlsson et al., 2009).

In contrast to such high antibiotic loads in effluents from antibiotic production companies, effluents from companies involved in the formulation of drugs contain much more modest antibiotic levels (typically <100 µg/L), however still being selective for AR (Šimatović and Udiković-Kolić, 2019; Bielen et al., 2017). The levels are still about one to two orders of magnitude higher than levels commonly detected in municipal effluents (low µg/L; Sabri et al., 2018, Michael et al., 2013), which were also shown to increase the abundance, diversity and potential spread of ARGs in recipient water bodies (Lekunberri et al., 2018; Khan et al., 2019; Corno et al., 2019). Further, often combinations of various antibiotics have been detected at sites from drug- formulation companies (Khan et al, 2013, Bielen et al, 2017); however, the effects of combined exposures of moderate levels of various antibiotics on environmental biota associated are far less explored.

In our previous study (Bielen et al., 2017), we showed that effluents from Croatian drug-formulation industry contained a range of antibiotics, including sulfonamides, tetracyclines and trimetoprim, in concentrations up to approx. 250 µg/L. More recently (González-Plaza et al., 2018, 2019), we also demonstrated that these effluents were sources of various ARGs (*sul*, *tet*, *dfr*, *fol*, *bla*), and significant amounts of culturable ARB, ARGs and MGEs such as broad host range IncP-1 plasmids and class 1 integrons. The aim of this study

was to investigate the effects of these formulation discharges on exposed creek sediments during the warm (summer) and the cold (winter) sampling conditions. We used chemical analyses of selected antibiotics, metals and nutrients to explore the pollution levels in the receiving creek sediments. The relative abundance of 15 ARG subtypes against 5 major antibiotic classes (sulfonamides, diaminopyridines, tetracyclines, ß-lactams and macrolides) was determined by quantitative PCR. Illumina-based 16S rRNA amplicon sequencing was applied to assess the impact on sediment bacterial community structure and network analysis was used to infer about potential bacterial hosts of increasing ARGs.

2. MATERIALS AND METHODS

2.1 Study area and sample collection

For this study samples were obtained from Kalinovica creek located in rural area in the northwest of Croatia, near the city of Zagreb, where the local drug-formulation facility discharges its wastewaters (Bielen et al., 2017; González-Plaza et al., 2018, 2019). This facility formulates various plant protection products and a wide range of drugs for human and veterinary use. The active pharmaceutical ingredients of these drugs are antibiotics mainly from sulfonamide, tetracycline, ß-lactam, diaminopyridine and macrolide classes. As previously described (Bielen et al., 2017), wastewaters leaving the pharmaceutical facility are a mixture of industry´s technological and sanitary wastewaters which have only undergone primary treatment, i.e. mechanical removing of larger, floating solids.

Sediment samples were collected from 3 locations along the creek over two sampling campaigns performed in winter (January, monthly average 0.8°C) and summer (July, monthly average 22.4°C) of 2016. The sampling sites were located 300 m upstream of the effluent discharge (reference site; UP), immediately at the effluent discharge site (DW0), and 3000 m downstream of the discharge (DW3000). Both UP and DW0 sites were situated in an agricultural area, whereas DW3000 was situated close to a forest. From each site, four replicates (approximately 500 g each) were collected within approximately 1-2 m apart from the surface of the sediment (0 – 5 cm) using a plastic core tube. Subsamples from each of the four replicate sediment samples (approximately 2 g) were stored at -80°C for DNA extraction, while the rest of the subsamples were composited (10 g of each subsample used) and air-dried at ambient temperature for physico-chemical analyses.

In addition to sediments, we used and analyzed the same effluent samples of the industry as described recently (Bielen et al., 2017; González-Plaza et al., 2018, 2019). Effluents 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, PA, USA) to collect the bacterial cells; filters were stored at -80°C until DNA extraction.

2.2. Physico-chemical analyses of sediments

Dry composite sediment samples were coarse grounded to < 2 mm. Physico-chemical properties, including pH, total organic carbon (TOC), total carbon (TC), total nitrogen (TN), total phosphorus (TP), nitrate, nitrite, and ammonia nitrogen were determined using internationally validated methods (ISO standards) as described previously (Milaković et al., 2019). Size fractions were measured with a Laser Coulter LS 13320 diffractometer (Beckman Coulter, USA).

2.3. Measurements of antibiotics and heavy metals in sediments

Four antibiotics belonging to three classes were analyzed in this study. The target sulfonamides included sulfadiazine (SDZ) and sulfamethazine (SMZ), the target diaminopyridine included trimethoprim (TMP), and the target macrolides included azithromycin (AZI). The antibiotics were extracted from the sediments using pressurized liquid extraction and analyzed by reversed-phase liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS) following the protocols previously described (Senta et al., 2008, 2013). The contents of heavy metals, including Cd, Cr, Cu, Pb, Ni, and Zn, in the sampled sediments were measured by inductively coupled plasma mass spectrometry (ICP-MS) as described previously (Dautović et al., 2014).

2.4. DNA extraction

DNA was extracted from wastewater filters and sediment cores for quantitative PCR and 16S rRNA gene sequencing using the Power Soil DNA isolation kit (MoBio, CA, USA) following the manufacturer´s recommendations. Non-template sample (DNA-free water) was included as a negative extraction control during the whole workflow. The quality of the extracted DNA was verified spectrophotometrically (BioSpec Nano, Shimadzu, Japan), while the quantity of DNA in samples was determined fluorometrically (Qubit Fluorometer 3.0, Thermo Fisher Scientific, USA). Extracted DNA was stored at -20°C until use.

2.5. Quantification of ARGs and 16S rRNA genes

Quantitative real-time PCR (qPCR) was conducted with extracted DNA to quantify ARGs conferring resistance to tetracyclines (*tet*C, *tet*39), ß-lactams (bla_{GES}, bla_{VEB}, bla_{OXA-1}, and bla_{OXA-2}), trimethoprim (*dfr*A14 and *fol*A), sulfonamides (*sul*1 and *sul*2) and macrolides (*mph*G, *mph*E, *msr*E, *mef*C, and *erm*B). In addition, copy number of the 16S rRNA gene (*rrn*) was determined to assess the total bacterial abundance and for normalization of the data. All qPCR assays were performed using a ABI 7300 Real-time PCR system (Applied Biosystems, CA, USA) and Power SYBR® Green PCR Master Mix (Applied Biosystems, CA, USA) in a total volume of 15 µL. Specific primer sets, annealing temperatures, amplification accuracies and efficiencies are listed in Table S1. Efficiency and accuracy values were determined from six points of the serial dilutions of each target ARG cloned into pGEM-Easy vector as previously described (Milaković et al, 2019). Thermal cycling conditions for all but three ARGs (i.e. bla_{OXA-1}, bla_{OXA-2}, and *erm*B) were as follows: 95 °C for 15 min, 30 cycles at 95 °C for 15s, annealing at corresponding temperature (Table S1) for 30s, and 72 °C for 30 s. For quantification of bla_{OXA-1} and bla_{OXA-2} genes, thermal cycling conditions were set in accordance to Zhai et al. (2016), for

quantification of *rrn* according to López-Gutiérrez et al. (2004), and for quantification of *erm*B in accordance with Chen et al. (2007). The quantification limit for all target ARGs was 10^2 gene copies per reaction. PCR inhibition test was performed with DNA diluted to 1 ng/µL and 0.1 ng/µL, following the procedure described previously (Petric et al., 2011); no inhibition was observed. To minimize the variance in bacterial concentration or amplification efficiency between samples, the relative gene abundance was calculated using the following equation: $\log_{10} 2^{(Ct\ rrn\ -\ Ct\ target\ ARG)}$.

2.5. Amplicon sequencing and data processing

We performed 16S rRNA gene amplicon sequencing to assess changes in bacterial community structure between the upstream and downstream sediments. The universal bacterial primer pair 27F and 357R covering the V1-V2 hypervariable region of the bacterial 16S rRNA gene was used (Klindworth et al., 2013). Amplicon libraries for sequencing were prepared following previously described method (Gschwendtner et al., 2016) with two modifications: samples were purified with NucleoSpin Gel and PCR Clean-Up kit (MACHEREY NAGEL GmbH & Co., Germany) during the library preparation; the size and concentrations of PCR products and fragments was validated using a FragmentAnalyzer instrument and the Standard Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, CA, USA). Subsequently, prepared amplicon libraries were sequenced on an Illumina Miseq Instrument (Illumina, United Kingdom, Chesterford) with the Miseq Reagent Kit v3 for 600 cycles.

Data processing included the removal of adapters by using AdapterRemoval tool (Schubert et al., 2016), analysis of the reads with the QIIME 2 v2018.2.0 (https://qiime2.org), and denoising using the DADA2 plugin (Callahan et al., 2016). Obtained amplicon sequencing variants (ASVs) were compared to the 99% identical clustered SILVA database v132 (Quast et

al., 2013), with a naive Bayes classifier trained on the amplified region. Alpha diversity was described for each sample by using metrics of observed species (i.e. ASVs), and rarefaction curves were generated to compare the level of bacterial ASVs diversity. Non-metric multidimensional scalling (NMDS) based on Bray-Curtis distance was performed with the Canoco software v5.1 to compare the overall composition of bacterial communities among different sampling sites and seasons. In addition, a heatmap visualisation of the relative abundance of genera that were significantly increased at DW sites compared to UP site was perfomed with the R Studio software v1.1.383 by using the heatmap.2 function within the 'gplots' package (Warnes et al., 2016).

2.6. Statistical analyses

Shapiro Wilk's test was applied on $\log_{10}$ transformed qPCR data ($2^{\wedge(Ct\ rrn\ -\ Ct\ target\ ARG)}$) to check whether our data follows the normal distribution with 'fitdistrplus' and 'stats' package in R studio v1.1.383. Kruskal-Wallis test was perfomed to determine differences between relative abundance of ARGs at each DW site and reference UP site. All statistical analyses were performed by using GraphPad Prism v6.01. To analyse the differences in the relative abundance of bacterial communities at phylum and genus level between UP and each DW site, the package DESeq2 v1.22.1 was applied (Jonsson et al., 2016; Love et al., 2014). All statistical tests were considered significant if adjusted $p < 0.05$ (Benjamini and Hochberg, 1995). Shannon-Wiener diversity index and Adonis test were performed using R studio sotware v1.1.383 with 'vegan'package. A correlation matrix was conducted with ARG and 16S rRNA data to explore the potential correlations among ARGs and bacteria (genus level) by calculating all pairwise Spearman's correlation coefficients (ρ) (Li et al., 2015). A correlation between two nodes was statistically significant if $\rho > 0.7$ and the p-value was < 0.01. To reduce

the chances of obtaining false-positive results, the *p*-values were adjusted by using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Network analysis was performed with R Studio v1.1.383 by using VEGAN (Oksanen et al., 2018), igraph (Csárdi and Nepusz, 2006) and Hmisc (Harrel and Frank, 2008) packages. Network visualisation was conducted in Cytoscape v3.7.0. (Shannon et al., 2003).

225 **3. RESULTS**

226 **3.1. Physico-chemical properties of sediments**

227 Physico-chemical properties of sediments from creek sampling are summarized in
228 Table S2. The sediment samples were slightly acidic to alkaline, with pH values ranging from
229 6.81 to 7.98. According to the particle grain-size, analyzed sediments were characterized as
230 silty-sand (Wenthworth, 1922), with silt and sand values ranging from 53 to 67% and 27 to
231 43%, respectively. The sediments from DW0 site had the lowest TOC (max 2.13%), TC (max
232 3.34%) and TN (max 0.17%) values over both seasons. In contrast, the maximum value of NO_3^-
233 (up to 24 mg/kg) was observed at this site during summer, and NH_4^+ (up to 16 mg/kg) during
234 winter season.

235 **3.2. Antibiotic concentration in creek sediments**

236 For the analysis of antibiotic concentrations in the creek sediments, sulfonamide,
237 diaminopyridine and macrolide antibiotics were chosen as the unique targets because they
238 are formulated into drugs by the pharmaceutical industry close by the creek and detected in
239 its wastewaters (Bielen et al., 2017). Despite some produced formulations may include
240 tetracyclines or ß-lactams, these compounds were not analyzed because quantitative method
241 for their determination in solid matrices was not established in our lab; however,
242 accumulation of tetracyclines is expected due to their constant input through effluent
243 discharge and the strong sequestration to organic matter (Huang et al., 2011). All target
244 antibiotics were detected in sediments from all sampling sites, being present in the lowest
245 levels (≤ 36 µg/kg) at UP during both seasons (Table 1). In contrast, the highest levels of TMP
246 (up to 5076 µg/kg) and AZI (up to 389 µg/kg) were detected at DW0 during both seasons,
247 particularly during summer. Despite the decrease in levels of these compounds at the more

distant site (DW3000), the antibiotics were still present in up to one order of magnitude higher higher amounts at DW3000 compared to UP. For sulfonamide antibiotics (SDZ and SMZ), the highest amounts were not found at site DW0 but at site DW3000 (total concentration 1175 µg/kg during winter and 561 µg/kg during summer, Table 1).

Table 1. Quantification of antibiotics belonging to three different classes over winter and summer season in creek sediments receiving drug-formulation effluents.

Antibiotic class	Antibiotic	Antibiotic abbreviation	Season	Sampling sites (µg/kg dry sediment)		
				UP	DW0	DW3000
Sulfonamides	Sulfadiazine	SDZ	Winter	7.30	258	693
			Summer	15	41	164
	Sulfamethazine	SMZ	Winter	32	195	482
			Summer	30	85	397
Diaminopyridines	Trimethoprim	TMP	Winter	36	371	275
			Summer	35	5076	301
Macrolides	Azithromycin	AZI	Winter	14	153	69
			Summer	14	389	355
Total antibiotics, ∑			Winter	89.3	977	1519
			Summer	94	5591	1217

Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

3.3. Heavy metal concentrations in sediments

Table 2 shows the concentration of heavy metals (Cd, Cr, Cu, Pb, Ni, and Zn) in sediments from the three investigated sampling sites. Compared to UP, DW0 showed slightly higher concentrations of Cr, Pb and Ni only during winter. Surprisingly, highest concentrations of both Cu and Zn were measured in sediments from UP during both seasons, especially of Zn (winter, 445 mg/kg and summer, 505 mg/kg), with a decrease of approximately 2 times (Zn) or 3 times (Cu) at DW0. Higher concentrations of both of these metals were found at DW3000 compared to DW0. It is important to emphasize that concentrations of both Cu and Zn at all sites were above the minimum co-selective concentrations (MCCs), i.e. concentration needed to co-select for metal and antibiotic resistance (Seiler and Berendonk, 2012).

Table 2. Concentration of heavy metals in sediments from different sites along the creek over winter and summer season.

Metal	MCC*	Season	Sampling sites (mg/kg of dry sediment)		
			UP	DW0	DW3000
Cadmium (Cd)	1.0	Winter	0.37	0.10	0.31
		Summer	0.42	0.24	0.63
Chromium (Cr)	-	Winter	111	139	74
		Summer	102	98	83
Copper (Cu)	11.5	Winter	**125**	**43**	**82**
		Summer	**171**	**45**	**134**
Lead (Pb)	-	Winter	46	63	39
		Summer	53	52	43
Nickel (Ni)	-	Winter	46	69	53
		Summer	178	35	35
Zinc (Zn)	42.5	Winter	**445**	**186**	**276**
		Summer	**505**	**207**	**396**

Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge. The values in bold represents 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).

3.4. Target ARGs in industrial effluents and creek sediments

We estimated the relative abundances of 15 ARGs in effluent and sediment samples over two seasons by using qPCR (Fig. 1). Among the analyzed ARGs in effluent samples, the most abundant genes in both seasons were sulfonamide (*sul*1 and *sul*2), macrolide (*mph*G and *msr*E), tetracycline (*tet*C and *tet*39*)*, and trimethoprim ARGs (*dfr*A14), with values mainly in the range of -1 to -2 log gene copies/*rrn* copies (Table S3; Fig. 1). However, the relative abundances of ß-lactam ARGs (bla_{GES}, bla_{VEB} and bla_{OXA-1}), except bla_{OXA-2} during summer, were in most cases 10-times lower (approx. -3 log gene copies/*rrn* copies), while the relative abundance of trimethoprim *fol*A and macrolide *mph*E, *mef*C and *erm*B subtypes was approximately 100-times lower (-4 log gene copies/*rrn* copies) (Table S3).

In creek sediments at UP site, during both seasons, the *sul*1, *dfr*A14 and *tet*C genes were detected at high abundances of approximately -1 to -2 log gene copies/*rrn* copies, while the relative abundances for *tet*39, bla_{VEB}, bla_{OXA-1}, bla_{OXA-2}, *sul*2 and *fol*A were in the range from -3 to -4 log gene copies/*rrn* copies (Fig.1, Table S3). Similar ARG levels (-3 log gene copies/*rrn* copies) were found for *mph*E and *mef*C during summer, and for *mph*G and *msr*E during winter. In contrast, the genes bla_{GES} and *erm*B were below quantification limit in UP sediment in both seasons (Table S3).

The discharge of industrial effluents differently affected the relative abundance of targeted ARGs in the receiving creek sediments (Fig. 1). During both seasons, the relative abundances of ARGs to ß-lactams, TMP, macrolides and sulfonamides significantly increased in sediments from DW0 compared to UP ($p < 0.05$; Kruskal-Wallis), with increases varying from only about one half to four orders of magnitude (Fig. 1, Table S3). Also, seasonal differences in the relative abundance of ARG subtypes were observed (Fig. 1 and Table S3). Specifically, among target ß-lactam ARGs, relative abundances of both bla_{GES} and bla_{OXA-1} subtypes

303 increased by one to three orders of magnitude at DW0 during both seasons, while *bla*$_{\text{OXA-2}}$
304 subtype was about one order of magnitude higher at DW0 compared to UP only during
305 summer. Regarding TMP resistance, *fol*A subtype was one order of magnitude higher in
306 relative abundance at DW0 compared to UP in winter, while only *dfr*A14 subtype was slightly
307 more abundant during summer (Fig. 1 and Table S3). Considering macrolide ARGs, *erm*B
308 subtype increased by two to three orders of magnitude at DW0 during both seasons, while
309 *mph*E and *mef*C were found elevated by up to four orders of magnitude only in winter. During
310 summer, *mph*G and *msr*E subtypes significantly increased in relative abundance (up to three
311 orders of magnitude) not only at DW0 but also at DW3000 compared to UP ($p < 0.05$; Kruskal-
312 Wallis; Fig. 1 and Table S3). Similar to this, the sulfonamide resistance gene *sul*2 increased in
313 relative abundance by up to two orders of magnitude at both DW0 and DW3000 compared to
314 UP in both seasons, whereas *sul*1 slightly increased (roughly half order of magnitude) at DW0
315 compared to UP only during winter. In contrast, tetracycline ARGs were found significantly
316 elevated during summer only at DW0 site, with increases of up to two orders of magnitude.
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318
319

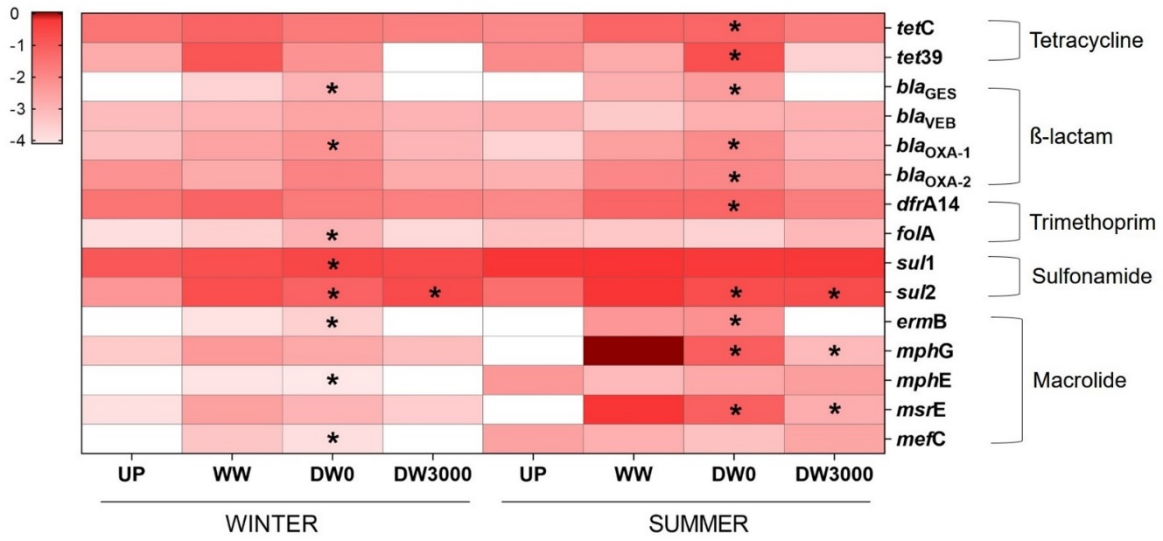


Figure 1. Heat map of relative abundances of 15 targeted ARGs in effluent (WW) and sediment samples taken from three sampling sites (UP, DW0, DW3000) over winter and summer season. Plotted values represent the natural logarithm-transformed the relative abundance of each ARG target (per 16S rRNA gene copy numbers). Asterisks represent statistically significant difference ($p < 0.05$, Kruskal-Wallis) between each DW and UP site. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

3.5. Impact of formulation effluents on sediment bacterial communities

In total, 3,425,195 bacterial raw-sequence reads were obtained from the PCR amplicon sequencing of a total of 27 effluent and sediment samples. After adapter removal, 3,425,140 high-quality bacterial sequence reads left covering bacterial V1-V2 hypervariable region, accounting for 2,265,504 high quality reads after denoising step. Those were assigned to a 13,461 ASVs at 99% similarity level. Rarefaction analysis showed that the sequencing depth of 27 datasets was sufficient to detect the most of the ASVs in the analyzed samples (Fig. S1).

Shannon-Wiener diversity index indicated that during both seasons discharge of pharmaceutical effluents had no significant effect ($p > 0.05$, Kruskal-Wallis) on overall bacterial

diversity in sediments from both DW0 and DW3000 sites in comparison with UP site (Fig. S2). However, NMDS analysis based on Bray-Curtis distance (Fig. 2) revealed that sediment samples from three studied sites (UP, DW0 and DW3000) formed three separate clusters (Adonis $R^2 = 0.8254$, $p < 0.05$), independent from sampling season.

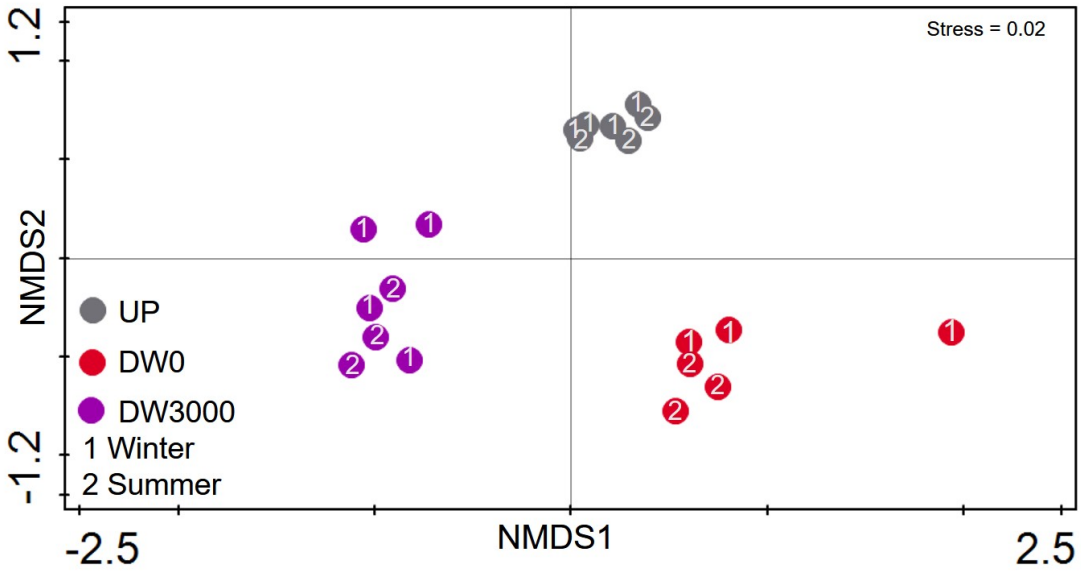
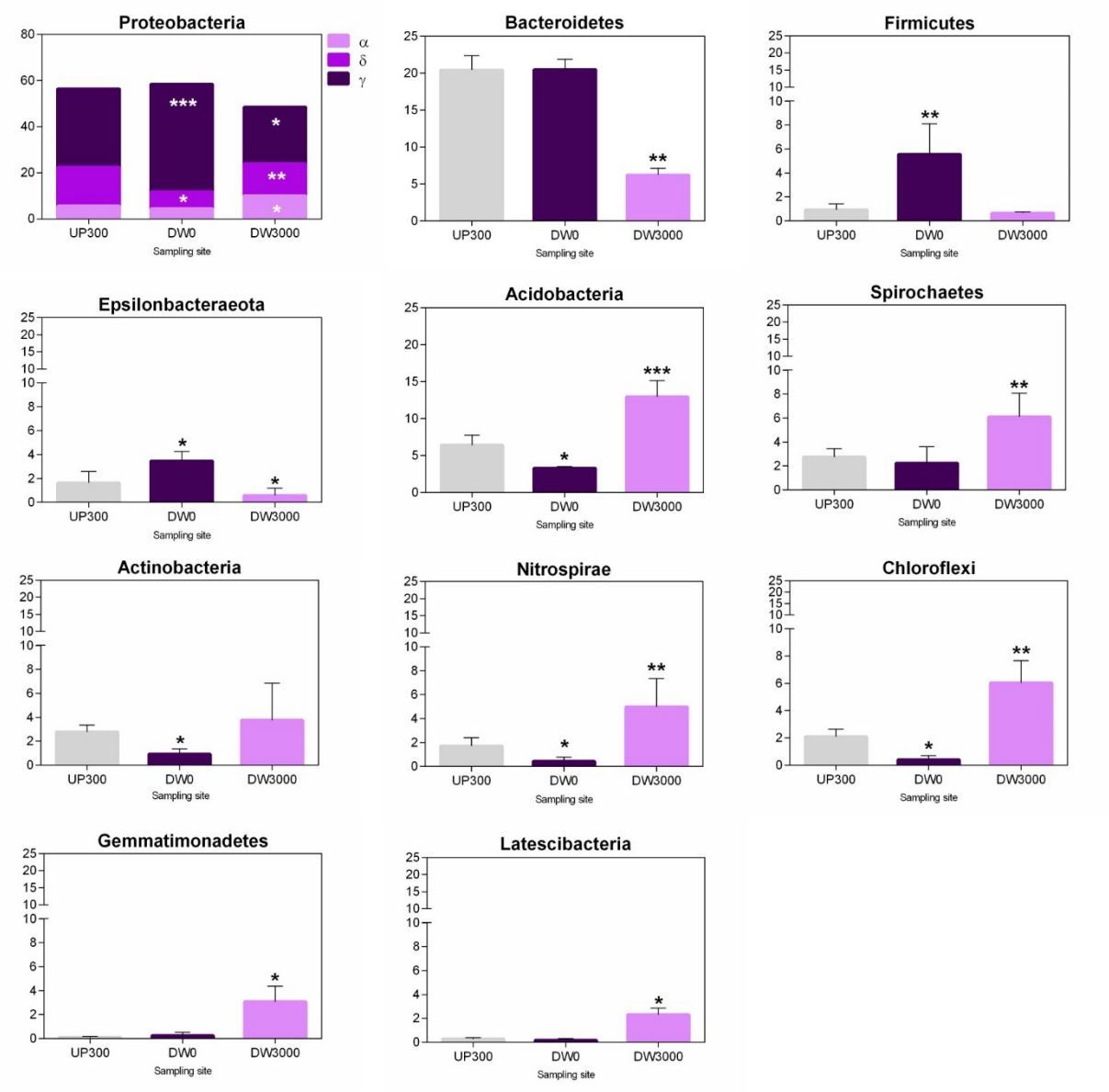


Figure 2. NMDS analysis based on Bray-Curtis distance showing the spatial changes in sediment community composition across three to four replicates of each of the three sites along the creek. The replicate samples from the same site has been marked with the same color, and from the same season with the same number. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

The comparison of the bacterial community composition in effluents and sediments (UP, DW0 and DW3000 sites) indicated that members of the phyla *Proteobacteria* and *Bacteroidetes* were the most abundant phyla in all samples during both seasons (Fig. S3). Other abundant phyla were *Acidobacteria* in all sediment samples, and *Firmicutes* and

Epsilonbacteraeota in both effluents and DW0 sediments. In addition, the dominant bacterial groups in DW3000 sediments were *Spirochaetes* and *Chloroflexi*.

Discharge of industrial effluents resulted in significant changes in the relative abundance of different phyla at DW0 and DW3000 compared to UP site as shown by DESeq2 analysis ($p < 0.05$) (Figs. 3 and S4). Compared to UP, the most significant increase in the relative abundance at DW0 during both seasons was observed for *Firmicutes* (5.2% two-season average) and *Epsilonbacteraeota* (2% two-season average). The proportion of these phyla decreased to background levels (*Firmicutes*) or significantly below background levels (*Epsilonbacteraeota*) at DW3000 (Figs. 3 and S4). Other phyla, such as *γ-Proteobacteria* showed significantly increased relative abundance at DW0 (12.5%) compared to UP only during winter, but again significantly decreased at DW3000 during both seasons. In contrast, the proportion of *Spirochaetes* significantly increased (5.7%) at DW0 only during summer, but was also significantly higher at DW3000 compared to UP during both seasons (winter 3.3%, summer 2.5%). The relative abundance of three phyla, i.e. *Acidobacteria*, *Chloroflexi* and *Nitrospirae*, significantly decreased at DW0 compared to UP, but was again increased at DW3000 for both seasons (Figs. 3 and S4). Moreover, a significant increase in relative abundance at DW3000 but not at DW0 compared to UP site was found for *α-Proteobacteria* (5.4% two-season average) as well as for *Gemmatimonadetes* and *Latescibacteria* (<3%) over both seasons.



370

371 **Figure 3.** Changes in bacterial community composition at phylum level in sediments from
372 different sites along the creek during winter season. The relative abundance of phyla is shown
373 based on their closest match to SILVA database at 99% similarity level. Asterisks represent a
374 significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; DESeq2) between each DW site and
375 UP site. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m
376 downstream of discharge.

377

The most abundant bacterial genera (>1%) in sediments from UP site in both seasons were affiliated with the families *Prolixibacteraceae* (BSV13, *Prolixibacter*, WCHB1-32), *Cyclobacteraceae*, *Ignavibacteriaceae* (*Ignavibacterium)*, *Geobacteraceae* (*Geobacter)*, *Archangiaceae (Anaeromyxobacter)*, *Steroidobacteraceae*, and *Spirochaetaceae* (*Sphaerochaeta)* (Tables S4 and S5). Discharge of antibiotic-formulation effluents induced significant changes in the relative abundance of different taxa in background sediment. To identify those taxa which were significantly increased in relative abundance ($p < 0.05$,) all ASVs at the genus level were subjected to DESeq2 analysis. In effluents, genera that were highly abundant (≥1%) in both seasons included *Acidovorax*, *Aeromonas*, *Pseudomonas*, *Acinetobacter (γ-Proteobacteria*), *Flavobacterium*, *Roseimarinus (Bacteroidetes)* and *Arcobacter* (*Epsilonbacteraeota*). Additionally, the genus *Rhodoferax (γ-Proteobacteria*) was found to be abundant only in winter effluents (Fig. 4a). All these genera were significantly increased in relative abundance in sediments from DW0 compared to UP but not at DW3000 in both seasons (Fig. 4, Tables S6 and S7). Besides, various other genera with low abundance in effluents (<0.1%) and UP sediment (≤0.8%) also showed significantly increased relative abundance in DW0 (>1%) compared to UP, with differences between seasons. For instance, in winter, *Sideroxydans* and *Luteimonas* (*γ-Proteobacteria*) showed higher relative abundances in DW0 compared to UP, whereas *Solobacterium* (*Firmicutes*) and *Treponema*2 (*Spirochaetes*) had higher abundances in DW0 compared to UP sediments only in summer (Fig. 4, Tables S6 and S7). In addition, some sediment-associated genera such as *Desulfobulbus* (δ-*Proteobacteria*) or effluent-associated *Thauera* (*γ-Proteobacteria*) were significantly increased in abundance at DW0 in both seasons (2% and 0.9% two-season average, respectively), whereas for *Smithella* (δ-*Proteobacteria*; sediment-associated) an increased abundance was only in summer (1.5%). Among all above-mentioned genera with enhanced relative

abundance at DW0, only the relative abundance of *Sideroxydans* (1.6% two-season average) and *Smithella* (1.4% summer) was still significantly increased at DW3000 compared to UP (Fig. 4, Tables S6 and S7). In addition, unassigned members of the family *Sphingomonadaceae* were found in significantly increased relative abundance at both DW0 and DW3000 sites compared to UP site during both seasons (0.6% two-season average).

There were few other taxa including *Azoarcus* and *Acidovorax* which were significantly increased in relative abundance at both DW sites compared to UP, but their proportion was <0.5%. Exception are *Azoarcus* (winter) and *Acidovorax* (summer) which had relative abundance of 1% and 5%, respectively at DW0 site (Fig. 4, Tables S6 and S7). Finally, the majority of genera that were significantly elevated and dominated (>3%) at downstream DW3000 site during both seasons, such as *Spirochaeta* 2, Ellin6067 group and *Nitrospira*, were present in low relative abundance (<0.7%) in DW0 sediment and originated mostly from UP sediments (Fig. 4, Tables S6 and S7). Notably, many of the taxonomic groups with significantly higher abundances at DW3000 compared to UP could not be classified to the genus level.

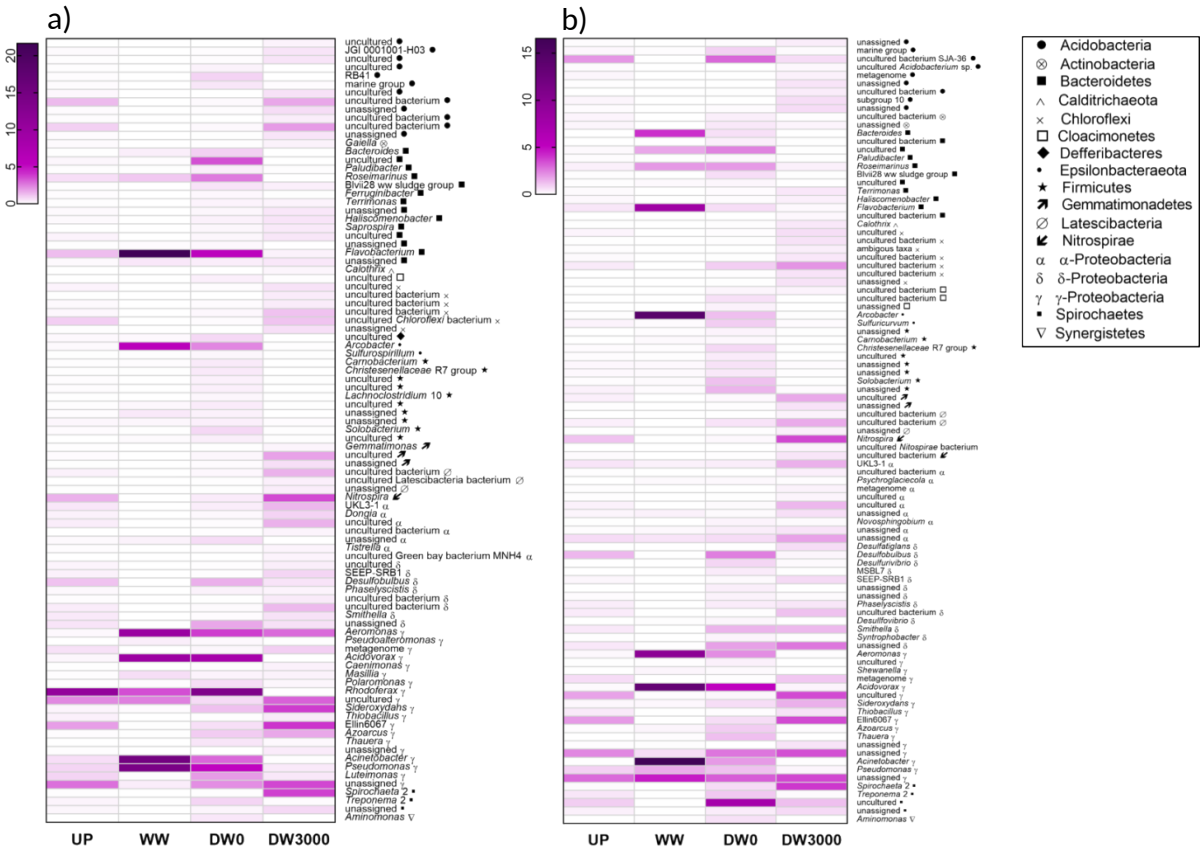
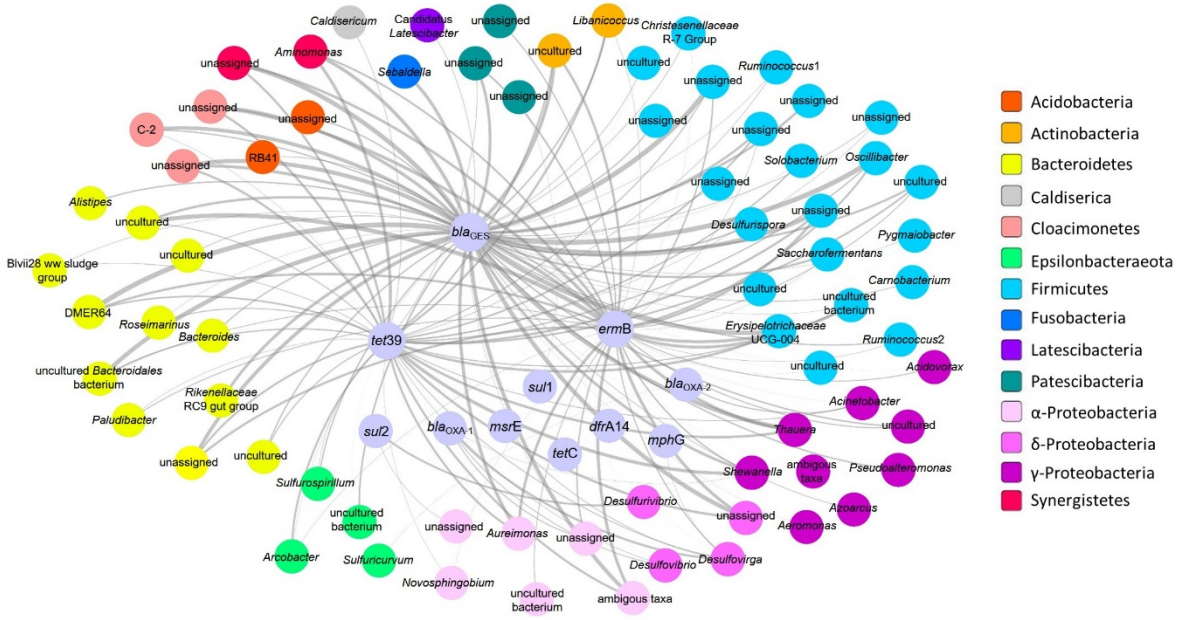


Figure 4. Heat maps showing relative abundance of genera that were significantly increased at DW sites compared to UP site ($p < 0.05$, DESeq2) during a) winter and b) summer season. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge; WW, effluent.

3.6. Co-occurrence between target ARGs and bacterial taxa

Network analysis was performed to identify potential bacterial taxa that might be associated with the analyzed ARGs (Fig. 5, Table S8). The entire network, consisting of 83 nodes and 155 edges, had a modular structure with a modularity index of 0.722 (Newman, 2006). Out of 15 ARGs targeted in this study, 11 were found to be significantly positively correlated with bacterial genera (bla_{GES}, bla_{OXA-1}, bla_{OXA-2}, *sul*1, *sul*2, *tet*39, *tet*C, *dfr*A14, *mph*G, *msr*E, and *erm*B). In total, the targeted ARGs had 72 potential bacterial hosts which mainly belonged to the *Firmicutes* (22), *Proteobacteria* [α- (6), δ- (4), γ- (9)], *Bacteroidetes* (12), and

Epsilonbacteraeota (4) (Fig. 1, Table S8). Regarding single ARG-host correlations, the gene bla_{GES} had the highest number of potential bacterial hosts (18), including *Azoarcus*, *Aeromonas* and members of uncultured family *Barnesiellaceae*, which were found to be highly abundant (≥1%) and increased at DW0 compared to UP (Fig. 5, Tables S6 and S7). The gene *sul*2 was the only one with increased abundance at both DW sites which showed significant correlations with ASVs of the family *Sphingomonadaceae* (*α-Proteobacteria*). However, among the multi ARGs-host correlations (at least 2 ARGs in individual host), the three ARGs, i.e. $bla_{GES,}$ *tet*39 and *erm*B co-occurred in the highest number of potential hosts (24), including those with significantly increased relative abundance at DW0 in both seasons, i.e. *Arcobacter*, *Thauera*, and *Aminomonas* (Fig. 5, Tables S6 and S7). Besides *erm*B, the genes bla_{GES} and *tet*39 co-occurred with bla_{OXA-2} in genera *Acinetobacter* and *Roseimarinus* which were increased and highly abundant (>1%) at DW0 but not at DW3000 in both seasons. In addition, the co-occurrence of four ARGs was found for *Shewanella* ($bla_{GES,}$ *tet*39, *erm*B and bla_{OXA-1}) and *Desulfovibrio* (*tet*39, *tet*C, bla_{OXA-2} and *dfr*A14), both significantly higher in abundance in summer at DW0 compared with UP (Fig. 5, Tables S6 and S7).



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447 **Figure 5.** Network analysis revealing co-occurrence patterns among analyzed ARGs and their
448 potential bacterial hosts. The nodes were coloured according to the phylum affiliation. A
449 connection represents strong (Spearman correlation coefficient (ρ) > 0.7) and significant (p <
450 0.01) correlation. Node size was weighted according to the number of connections (i.e.
451 degree) and edges were weighted according to the correlation coefficient.

4. Discussion

The present study provides a comprehensive dataset on the effects of discharges of partially-treated effluents from Croatian drug-formulation pharmaceutical industry on the sediments from the receiving creek.

4.1. Contribution of industrial waste to antibiotic, metal and nutrient pollution of the receiving creek sediments

We showed that industrial discharges contributed to antibiotic accumulation in creek sediments, with levels typically highest at the discharge site for both trimethoprim (up to 5076 µg/kg) and azithromycin (up to 389 µg/kg), whereas the total concentration of two sulfonamides was the highest at the site located 3 km downstream (up to 1175 µg/kg). This accumulation of sulfonamides might be the result of the slower flow rate of the creek at DW3000 compared to DW0 , which in turn might accelerate the sedimentation of antibiotics in the sediment (González-Plaza et al., 2019). Additionally, sulfonamides are liable to degradation by sunlight (Baena-Nogueras et al., 2017) and the forest around DW3000 may have protected them from potential photodegradation resulting in their greater persistence in sediments at DW3000 versus DW0. Further, total antibiotic levels measured in the present study (up to 5 mg/kg at DW0 and 1.5 mg/kg at DW3000) were lower than what is generally found in sediments impacted by discharges from antibiotic production (tens of mg/kg) (Gothwal and Shashidhar, 2017; Kristiansson et al., 2011; Milaković et al., 2019), but higher than levels found in sediments exposed to treated effluents from municipal wastewater treatment plants (WWTPs) (up to 600 µg/kg total) (Li et al., 2019; Guang et al., 2019; Marti et al., 2014). In addition to antibiotics, we found that formulation effluents also contributed to a slight accumulation of nutrients, especially N compounds at DW0 which may affect the

composition of bacterial communities (Ibekwe et al., 2016). Some metals were also found to be elevated at DW0 compared to UP; however, surprisingly, the majority of targeted metals, especially Cu and Zn, were found in higher concentrations at UP than at both DW sites, suggesting other sources of contamination at UP. Importantly, Cu and Zn levels at UP and DW sites could co-select for AR (Seiler and Berendonk, 2012). Besides metals, there were also relatively low levels of antibiotics in UP sediments (up to 36 µg/kg). Given that studied creek flows through rural area without sewage treatment infrastructure, we speculate that untreated household waste disposal and agricultural runoff might be sources of pollution of UP sediments with antibiotics and metals.

4.2. Effects on antibiotic resistance genes in exposed sediments

Besides introducing antibiotics (Bielen et al., 2017), we showed in this study that formulation effluents in both seasons also introduced relatively high amounts (> -2 log gene copies/*rrn*) of the ARGs conferring resistance to sulfonamides (*sul*1 and *sul*2), tetracyclines (*tet*39, *tet*C), macrolides (*mph*G and *msr*E), and trimethoprim (*dfr*A14). The relative abundance of almost all these ARG subtypes significantly increased in the sediment at DW0 compared to UP during summer, but not during winter (except the *sul* ARGs). This seasonal difference may be linked to the warmer temperatures which may promote the survival of effluent-associated bacteria carrying ARGs or horizontal gene transfer (HGT) in sediments (González-Plaza et al., 2019), and thus lead to a increased relative abundance of ARG, despite the relatively high ARG abundance already present in the background sediments (UP site). In addition to the above-mentioned highly abundant ARGs, effluents also contained moderate amounts (-3 to -4 log ARG copies/*rrn*) of various ARG subtypes encoding resistance to ß-lactams (bla_{GES}, bla_{OXA-1}, and bla_{OXA-2}), trimethoprim (*fol*A) and macrolides (*erm*B, *mph*E, and

498 *mef*C). Most of these ARGs, except *erm*B and bla_{GES}, showed different dynamics during winter
499 and summer sampling, but were always higher in relative abundance at DW0 compared to UP.
500 These differences between seasons may be explained by variations in background sediment
501 levels of analyzed ARGs.

502 *erm*B and bla_{GES} ARGs, which are of high relevance in clinical settings (Guo et al., 2018;
503 Ohkoshi et al., 2018; Wibberg et al., 2018) were below quantification in UP sediment, but
504 found elevated at DW0 site during both seasons, suggesting deposition from incoming
505 industrial effluents. Indeed, both genes were measured in analyzed industrial effluents in
506 concentrations of -3 to -4 log gene copies/*rrn* copies which is comparable to or even lower
507 than concentrations previously found in municipal effluents (-2 to -3 log gene copies/*rrn*
508 copies) (Rodriguez-Mozaz et al., 2015; Rafraf et al., 2016; Yang et al., 2016). As a consequence,
509 the enrichment of these genes was also reported in sediments exposed to treated (*erm*B gene;
510 Sabri et al., 2018) or untreated municipal effluents (bla_{GES}; Marathe et al., 2017). The *erm*B
511 gene was also reported to be enriched in sediments exposed to drug-formulation effluents in
512 Pakistan (Khan et al., 2013).

513 However, most of ARGs with increased abundance in sediments at DW0 did dissipate
514 to background levels at the more distant DW3000 site. These results indicate either limited
515 transport / death of bacterial hosts (Milaković et al., 2019), degradation of extracellular DNA
516 containing ARGs (Nnadzoie and Odume, 2019), binding of ARGs to sediment (Calero-Cáceres
517 et al., 2017), or a combination. Nevertheless, three gene subtypes, i.e. *sul*2, *mph*G and *msr*E,
518 were detected significantly elevated above background also at DW3000 site. This might be
519 due to growth of their hosts as a result of selection pressure from residual antibiotics
520 (particularly sulfonamides) or expansion of hosts due to HGT, rather than transport of fecal

bacteria from DW0. The latter cannot be entirely excluded, although the relative abundance of taxa from the orders of *Bacteroidales* and *Clostridiales*, typically associated with fecal contamination (Halliday et al., 2014, McLellan et al., 2010), was low at DW3000 site (Tables S4 and S5). In contrast, the hyothesis for HGT is further supported by a previous study reporting the selection of *sul*2-carrying population in soil via HGT already at SDZ concentrations of 150 µg/kg (Heuer et al., 2008), which is lower than 164 µg SDZ/kg (summer) and 693 µg SDZ/kg (winter), measured in DW3000 sediments in this study. In addition, the selective concentrations of macrolides in the sediment are currently unknown, and thus, it is difficult to estimate whether sediment levels of AZI measured at DW3000 site (355 µg/kg) were selective for bacteria carrying *mph*G and *msr*E genes or increased HGT for these genes. Alternatively, increased relative *mph*G and *msr*E abundance at DW3000 may be a result of co-selection by sulfonamides. Co-localization of *mph*G and *sul*2 ARGs on the same genetic element further supports this assumption (González-Plaza et al., 2017; Nonaka et al., 2012).

4.3. Effects on bacterial communities in creek sediments

The community analysis revealed small difference in the number of taxa between the UP and downstream sediment samples, suggesting no impact of industrial pollution on sediment biodiversity. A similar conclusions was reached in other studies investigating the impact of antibiotics discharged from manufacturing facilities on exposed communities (Milaković et al., 2019; Bengtsson-Palme et al., 2019; Kristiansson et al., 2011). However, we observed clear effects of effluent discharge on the sediment bacterial community composition as the NDMS analysis revealed separate groups for UP, DW0 and DW3000 sediments, regardless of the season. Interestingly, DW0 where the industrial effluent is mixed with the creek water seemed to be taxonomically (phylum-level) more similar to effluent rather than

to UP or DW3000 sediments. The relative abundance of *Firmicutes* and *Epsilonbacteraeota*, which were among the most abundant phyla in the analyzed effluents, but also in pharmaceutical effluents described previously (Li et al., 2010, Marathe et al., 2013; Milaković et al., 2019), were significantly increased at DW0 site in both seasons, suggesting a deposition of effluent-associated bacteria in sediments close to the effluent outfall. At the downstream DW3000 site, these phyla were significantly reduced in proportion (< 1%), likely due to die-off or lack of transport of effluent-originating bacteria. In contrast, phyla more abundant in sediments than in effluents such as *Acidobacteria*, *Chloroflexi*, *Nitrospirae*, *Gemmatimonadetes*, *Latescibacteria* and *α-Proteobacteria* were increased in relative abundance at DW3000, but not at DW0 compared to UP site. Such distinct community composition at DW3000 site compared to both UP and DW0 sites may potentially be due to selection imposed by high concentration of antibiotics (total >1.2 mg/kg in both seasons) and other co-existing pollutants including heavy metals. However, other environmental factors including nutrients and habitat alterations can contribute as well.

Since the bacterial community has been identified as one of the key drivers that shape the ARG profiles in antibiotic-rich environments (Forsberg et al., 2014; Su et al., 2015), we performed network analysis in order to link variation of analyzed ARGs with the dynamic of the bacterial community. We found an association between some clinically relevant ARGs and ASVs more abundant at effluent-receiving sediments. For instance, *Azoarcus* and *Aeromonas* were found to host clinically relevant ß-lactam bla_{GES} subtype, while *Sulfuricurvum* mainly carried bla_{OXA-2}. Previous studies reported localization of bla_{GES} on plasmid in *Aeromonas* spp. isolated from rivers (Girlich et al., 2014) and from urban WWTP (Piotrowska et al., 2017), suggesting that waterborne *Aeromonas* species can be important reservoirs and vehicles for dissemination of GES-type ß-lactamases in the environment. Some taxa took along 3-4 ARGs

568 including *Arcobacter* (*bla*$_{GES}$, *tet*39, *erm*B), *Acinetobacter* (*bla*$_{GES}$, *bla*$_{OXA-2}$, *tet*39) or *Shewanella*
569 (*bla*$_{GES}$, *bla*$_{OXA-1}$, *tet*39, *erm*B). The last three genera together with *Aeromonas* had been
570 considered as the opportunistic human and/or animal pathogens (Janda, 2014; Wong et al.,
571 2017; Ferreira et al., 2015; Janda and Abbott, 2010), suggesting that industrial effluent
572 discharge increased the prevalence of pathogens carrying multiple ARGs of clinical relevance
573 in the receiving creek sediments. This may increase the risk of direct transmission of these
574 multiresistant pathogens to humans. However, for ARGs with higher prevalence at DW3000
575 versus UP site (*sul*2, *mph*G and *msr*E), we found an association of only *sul*2 with ASVs of the
576 family *Sphingomonadaceae* which were more abundant at both DW sites than at UP site. This
577 family has already been linked with sulfonamide resistance (Narciso da Rocha et al., 2014; Vaz-
578 Moreira et al., 2011) and assumed for being prone for acquiring *sul* genes (Narciso da Rocha
579 et al., 2014).

580 **5. CONCLUSIONS**

581 The present study revealed that effluent discharges from local drug-formulation facility
582 contributed to pollution of the receiving creek sediments with antibiotics and ARGs despite
583 relatively high background levels of the investigated genes in the creek. In addition, regardless
584 of the season, effluent discharge caused pronounced changes in sediment bacterial
585 communities from both downstream sites compared to upstream, but the overall taxonomic
586 diversity was not affected. In contrast to effluent discharge site where increased levels of
587 analyzed ARGs are likely a consequence of deposition of effluent-associated bacteria, the
588 accumulated levels of sulfonamides at more distant downstream site could play a role in
589 shifting community composition and increasing some sulfonamide and macrolide ARGs. Our
590 results demonstrate the necessity for implementing/improving infrastructure for the

591 treatment of sewage and industrial waste in the analyzed region in order to limit
592 environmental transmission of antibiotic residues and antibiotic resistance determinants.

593 **Data accessibility**

594 The 16S rRNA gene sequences that supports the findings of this study have been deposited in
595 the GenBank within the BioProject with the accession code ---.

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601 **Declaration of interest**

602 None.

603 **Supplementary data**

604 Supplementary data to this article can be found online at ---.

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Declaration of interests

 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Supplementary material for the manuscript entitled

Effects of industrial effluents containing moderate levels of antibiotic mixtures on the abundance of antibiotic resistance genes and bacterial community composition in exposed creek sediments

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Table S1. Primer pairs and conditions used to quantify 16S rRNA and antibiotic resistance genes targeted in this study.

Target gene	Resistance phenotype	Primer sequence 5' → 3'	Amplicon size (bp)	Tm* (°C)	Amplification accuracy/efficiency	Reference
***tet*C**	Tetracyclines	GGCATTCTGCATTCACTCGC GAAGCAAGCAGGACCATGATC	170	60	0.996 / 107.24%	This study**
***tet*39**	Tetracyclines	GCGGGAATTACAGGTGCCAAC GCAGCAAAGAACGGTGCATG	182	60	0.991 / 98.24%	This study**
***bla*$_{GES}$**	Beta-lactams	GCGGGTTTTCCTAAAGATTGG AATAACTTGACCGACAGAGGC	196	65	0.994 / 90.15%	Gatica et al., 2016
***bla*$_{VEB}$**	Beta-lactams	CGACTTCCATTTCCCGATGC CCAATATTGTCGCTCTCTGATAC	183	65	0.996 / 82.19%	Naas et al., 2000 (F)/This study** (R)
***bla*$_{OXA-1}$**	Beta-lactams	TATCTACAGCAGCGCCAGTG CGCATCAAATGCCATAAGTG	199	60	0.999 / 95.89%	Yang et al., 2012
***bla*$_{OXA-2}$**	Beta-lactams	TCTTCGCGATACTTTTCTCCA ATCGCACAGGATCAAAAACC	177	60	0.999 / 99.26%	Yang et al., 2012
***dfr*A14**	Trimethoprim	GTCGTTACCCGCTCAGGTTG GTCGATCGTCGATAAGTGGAG	177	67	0.992 / 104.12%	This study**
***fol*A**	Trimethoprim	CGAAGCTCCAGAAGGTTGTTTG CCGCTTCAATCGTAGTATGCAC	167	67	0.990 / 100.05%	This study**
***sul*1**	Sulfonamides	CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT	965	65	Heuer and Smalla, 2007	Heuer and Smalla, 2007
***sul*2**	Sulfonamides	CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT	865	65	Heuer et al., 2008	Heuer et al., 2008
***mph*G**	Macrolides	GGTATAAGTGAGCAATTGGAAAC GCTCCATCCTTTGAAGCTAG	128	60	0.998 / 105.39%	Milaković et al., 2019
***mph*E**	Macrolides	CTGTTTTCGGTGAAGAAAGTC CCATAAGCTAGAGGTGCGG	124	60	0.999 / 97.25%	Milaković et al., 2019
***msr*E**	Macrolides	CAATGTTATCTCGCCTTGGTG GTTGGTTCATCCGCTAGAATG	127	60	0.998 / 99.87%	Milaković et al., 2019
***mef*C**	Macrolides	GCTTACAAGTTATGCTGTTCAG CAGAGAGCTATAAAAGCATCC	195	60	0.996 / 104.37%	Milaković et al., 2019
***erm*B**	Macrolides	GATACCGTTTACGAAATTGG GAATCGAGACTTGAGTGTGC	364	58	0.990 / 105.15%	Chen et al., 2007
16S rRNA		CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGCA	196	60	0.993 / 83.64%	López-Gutiérezz et al., 2004

'Resistance phenotype' denotes the classes of antibiotics to which the gene product confers resistance to.

* annealing temperature

** primers were constructed based on sequences of previously detected antibiotic resistance genes (González-Plaza et al., 2018) by using the MEGA software (version 7.0)

Table S2. Physico-chemical properties of sediments at sampling sites along the creek over winter and summer sampling campaign.

Parameter	Season	Sampling sites		
		UP	DW0	DW3000
pH (H_2O)	winter	7.02	7.93	7.21
	summer	7.62	7.98	6.81
T (°C)	winter	10.4	6.9	3.0
	summer	22.2	20.7	23.0
Conductivity (25°C) (µS/cm)	winter	198.0	144.1	175.2
	summer	283.0	309.0	386.0
Sand (%)	winter	26.6	27.1	26.6
	summer	30.2	29.7	43.0
Silt (%)	winter	67.0	63.5	66.9
	summer	64.9	64.6	53.4
Clay (%)	winter	6.4	9.4	6.5
	summer	4.9	5.7	3.6
Total organic carbon, TOC (%)	winter	3.37	1.79	3.41
	summer	3.93	2.13	6.23
Total carbon, TC (%)	winter	4.13	2.14	5.05
	summer	4.74	3.34	6.32
Total nitrogen, TN (%)	winter	0.29	0.17	0.30
	summer	0.39	0.25	0.48
Total phosphorus, TP (%)	winter	0.09	0.03	0.06
	summer	0.11	0.09	0.10
NO_2^- (mg/kg)	winter	<0.3	<0.3	<0.3
	summer	<0.3	<0.3	<0.3
NO_3^- (mg/kg)	winter	22.8	21.0	11.0
	summer	19.0	24.0	19.0
NH_4^+ (mg/kg)	winter	9.77	16	<0.4
	summer	55.0	23.0	21.0

Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3 000 m downstream of discharge.

Table S3. Average relative abundance [$\log_{10} 2^{\wedge(Ct\ rrn\ -\ Ct\ target\ ARG)}$] of targeted ARGs in drug-formulation effluents (WW) and sediments of the receiving creek over winter and summer sampling season.

Target gene	Sampling campaign	Creek sampling site			
		UP	WW	DW0	DW3000
***tet*C**	winter	-1.45	-0.92	-1.41	-1.60
	summer	-1.91	-1.17	-0.21	-1.67
***tet*39**	winter	-2.74	-0.69	-1.84	<LOQ
	summer	-2.41	-2.68	-0.71	-3.56
bla_{GES}	winter	<LOQ	-3.42	-2.55	<LOQ
	summer	<LOQ	-2.82	-2.39	<LOQ
bla_{VEB}	winter	-3.09	-2.85	-2.36	-2.78
	summer	-2.84	-3.41	-2.86	-2.90
bla_{OXA-1}	winter	-2.99	-2.10	-1.52	-2.75
	summer	-3.62	-2.49	-2.01	-2.90
bla_{OXA-2}	winter	-2.14	-2.03	-1.77	-1.91
	summer	-2.86	-1.97	-1.82	-2.56
***fol*A**	winter	-3.88	-3.45	-2.45	-3.82
	summer	-3.26	-3.38	-3.87	-2.83
***dfr*A14**	winter	-1.45	-0.95	-1.41	-1.60
	summer	-1.88	-1.17	-1.21	-1.67
***sul*1**	winter	-0.89	-0.69	-0.50	-0.60
	summer	-0.11	-0.07	-0.21	-0.17
***sul*2**	winter	-2.30	-0.60	-0.66	-0.46
	summer	-1.38	-0.13	-0.65	-0.56
***mph*G**	winter	-2.43	-1.96	-2.27	-2.71
	summer	<LOQ	0.04	-1.13	-2.92
***mph*E**	winter	<LOQ	-3.83	-3.65	<LOQ
	summer	-2.25	-3.07	-2.70	-2.44
***msr*E**	winter	-2.71	-1.96	-2.51	-3.04
	summer	<LOQ	-0.10	-1.04	-2.63
***mef*C**	winter	<LOQ	-3.10	-3.58	<LOQ
	summer	-2.48	-2.86	-2.89	-2.60
***erm*B**	winter	<LOQ	-3.72	-3.00	<LOQ
	summer	<LOQ	-2.28	-2.15	<LOQ

<LOQ – Below limit of quantification. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

Table S4. Relative abundance of taxa (%) typically present at UP site compared to DW sites or effluent (WW) over winter season (DESeq2, $p < 0.05$).

Phylum	Class	Order	Family	Genus	Sampling sites			
					UP	WW	DW0	DW3000
Acidobacteria	Subgroup 18	Uncultured Acidobacteria bacterium			0.37±0.11	0.00±0.00	0.00±0.00	0.20±0.16
	Holophagae	Subgroup 7	Uncultured bacterium SJA-36		1.17±0.22	0.00±0.00	0.93±0.37	0.82±0.13
Actinobacteria	Actinobacteria	Unassigned	Unassigned	Unassigned	0.34±0.06	0.00±0.00	0.04±0.02	0.14±0.22
		PeM15	metagenome		0.19±0.05	0.00±0.00	0.00±0.00	0.02±0.04
	Coriiobacteria	OPB41	Uncultured bacterium		0.25±0.08	0.00±0.00	0.03±0.03	0.03±0.02
			Unassigned		0.18±0.03	0.00±0.00	0.01±0.01	0.00±0.01
	Acidimicrobiia	Microtrichales	Ilumatobacteraceae	CL500-29 marine group	0.13±0.02	0.00±0.00	0.01±0.02	0.08±0.07
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidetes BD2-2	Uncultured bacterium	0.51±0.20	0.00±0.00	0.00±0.00	0.01±0.02
			Unassigned	Unassigned	1.00±0.17	0.22±0.06	0.37±0.03	0.38±0.13
			Prolixibacteraceae	BSV13	2.70±0.50	0.00±0.00	0.73±0.37	0.13±0.08
				Prolixibacter	1.12±0.19	0.00±0.00	0.00±0.00	0.13±0.04
				WCHB1-32	3.67±0.57	0.93±0.10	1.38±0.78	0.16±0.14
				Uncultured	2.70±0.38	0.00±0.00	0.03±0.01	0.22±0.09
				Unassigned	1.16±0.20	0.10±0.02	0.08±0.10	0.34±0.07
		Cytophagales	Cyclobacteraceae	*Ekhidna*	0.39±0.11	0.02±0.01	0.33±0.40	0.17±0.09
				Unassigned	1.67±0.28	0.02±0.00	0.23±0.24	0.06±0.07
	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	*Ignavibacterium*	1.03±0.15	0.00±0.00	0.02±0.03	0.29±0.19
Chloroflexi	Anaerolineae	SJA-15	Ambigous taxa		0.41±0.18	0.00±0.00	0.00±0.00	0.04±0.01
Cyanobacteria	Oxyphotobacteria	Chloroplast	Coelastrella sp. M60		0.28±0.12	0.00±0.00	0.00±0.00	0.00±0.00
Epsilonbacteraeota	Campylobacteria	Campylobacterales	Thiovulaceae	*Sulfuricurvum*	1.50±0.92	0.01±0.01	0.75±0.35	0.53±0.54
Nitrospinae	P9X2b3D02	Uncultured bacterium			0.49±0.17	0.00±0.00	0.00±0.00	0.04±0.05
Proteobacteria	α-Proteobacteria	Acetobacterales	Acetobacteraceae	*Rhodovastum*	0.12±0.04	0.00±0.00	0.02±0.03	0.00±0.00
				Roseomonas	0.58±0.07	0.00±0.00	0.15±0.10	0.17±0.02
		Rhodobacterales	Rhodobacteraceae	*Planktomarina*	0.18±0.06	0.01±0.01	0.10±0.04	0.10±0.13
				Unassigned	0.57±0.21	0.30±0.01	0.18±0.12	0.06±0.07
	δ-Proteobacteria	Deltaproteobacteria Inc. Sedis	Syntrophorhabdaceae	*Syntrophorhabdus*	0.47±0.16	0.00±0.00	0.01±0.01	0.35±0.25
		Desulfobacterales	Desulfobacteraceae	[*Desulfobacterium*] catecholicum group	0.27±0.05	0.00±0.00	0.02±0.01	0.05±0.04
		Desulfuromonadales	Geobacteraceae	*Geobacter*	8.63±0.88	0.00±0.00	2.62±0.84	2.56±2.64
				Geothermobacter	0.90±0.19	0.00±0.00	0.02±0.01	0.06±0.05
			Sva1033	Uncultured bacterium	0.22±0.10	0.00±0.00	0.00±0.00	0.07±0.05

		Myxococcales	Archangiaceae	*Anaeromyxobacter*	1.14±0.26	0.00±0.00	0.15±0.22	0.50±0.26
			MidBa8	Uncultured deltaproteobacterium	0.28±0.09	0.00±0.00	0.01±0.01	0.01±0.01
	γ-Proteobacteria	Betaproteobacteriales	Burkholderiaceae	Unassigned	7.85±1.39	4.08±0.29	2.56±1.30	1.02±0.52
			Rhodocyclaceae	*Sulfuritalea*	0.31±0.12	0.00±0.00	0.02±0.04	0.25±0.18
		SAR86 clade	Uncultured marine eukaryote		0.24±0.07	0.00±0.00	0.04±0.04	0.10±0.06
		Steroidobacterales	Steroidobacteraceae	Uncultured	1.74±0.21	0.00±0.00	0.39±0.26	1.40±0.62
		Unassigned	Unassigned	Unassigned	2.11±0.29	0.05±0.01	0.57±0.76	1.33±0.28
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Sphaerochaeta*	2.12±0.80	0.00±0.00	0.01±0.01	0.35±0.23

Table S5. Relative abundance of taxa (%) typically present at UP site compared to DW sites or effluent (WW) over summer season (DESeq2, $p < 0.05$).

Phylum	Class	Order	Family	Genus	Creek sampling sites			
					UP	WW	DW0	DW3000
Acidobacteria	Subgroup 6	Uncultured bacterium	Unassigned	Unassigned	1.84±0.25	0.00±0.00	0.10±0.03	1.42±0.19
	Subgroup 18	Uncultured Acidobacteria bacterium			0.47±0.15	0.00±0.00	0.02±0.03	0.10±0.03
Actinobacteria	Actinobacteria	Catenulisporales	Actinospicae	*Actinospica*	0.13±0.04	0.00±0.00	0.02±0.02	0.05±0.04
		PeM15	metagenome		0.15±0.07	0.00±0.00	0.00±0.00	0.04±0.02
		Unassigned	Unassigned	Unassigned	0.35±0.09	0.01±0.01	0.09±0.01	0.06±0.05
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidetes BD2-2	Uncultured bacterium	0.38±0.05	0.00±0.00	0.00±0.00	0.00±0.00
			Prolixibacteraceae	BSV13	4.22±0.76	0.02±0.01	1.18±0.52	0.04±0.01
				Prolixibacter	1.33±0.24	0.00±0.00	0.05±0.06	0.23±0.03
				WCHB1-32	4.03±0.50	10.96±1.11	2.31±0.03	0.05±0.04
				Uncultured	3.22±0.66	0.00±0.00	0.11±0.09	0.26±0.12
				Unassigned	1.05±0.22	0.00±0.01	0.12±0.09	0.35±0.05
			Unassigned	Unassigned	0.95±0.19	0.08±0.02	0.28±0.20	0.47±0.11
		Cytophagales	Cyclobacteriaceae	*Ekhidna*	0.13±0.07	0.00±0.00	0.01±0.01	0.05±0.05
				Unassigned	1.25±0.11	0.00±0.00	0.02±0.02	0.05±0.04
			Microscillaceae	Uncultured	0.55±0.34	0.00±0.00	0.03±0.01	0.06±0.01
		Flavobacteriales	Unassigned	Unassigned	0.13±0.04	0.01±0.01	0.02±0.02	0.01±0.02
	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	*Ignavibacterium*	1.43±0.23	0.00±0.00	0.03±0.02	0.18±0.09
Chloroflexi	KD4-96	Uncultured Chloroflexi bacterium			0.81±0.11	0.00±0.00	0.10±0.06	0.13±0.07
	SJA-15	Ambigous taxa	Unassigned		0.19±0.07	0.00±0.00	0.02±0.02	0.02±0.00
Cyanobacteria	Oxyphotobacteria	Chloroplast	Coelastrella sp. M60	Unassigned	0.18±0.04	0.00±0.00	0.03±0.04	0.00±0.00
Nitrospinae	P9X2b3D02	Uncultured bacterium			0.28±0.12	0.00±0.00	0.00±0.00	0.03±0.03
Proteobacteria	α-Proteobacteria	Acetobacterales	Acetobacteraceae	*Roseomonas*	0.53±0.10	0.01±0.01	0.15±0.10	0.26±0.06
				Rhodovastum	0.14±0.01	0.00±0.00	0.01±0.02	0.05±0.01
	δ-Proteobacteria	Desulfuromonadales	Geobacteraceae	*Geobacter*	9.88±1.75	0.01±0.01	9.35±1.69	1.39±0.66
				Geothermobacter	0.96±0.32	0.00±0.00	0.02±0.02	0.09±0.06
		Myxococcales	Archangiaceae	*Anaeromyxobacter*	1.46±0.12	0.00±0.00	0.12±0.03	0.47±0.21
			MidBa8	Uncultured deltaprotebacterium	0.47±0.10	0.00±0.00	0.02±0.02	0.01±0.00
	γ-Proteobacteria	Betaproteobacteriales	Burkholderiaceae	*Rhodoferax*	9.40±1.18	2.73±0.09	8.95±1.32	3.74±1.24
			Unassigned	Unassigned	4.22±0.63	1.19±0.05	0.97±0.65	1.11±0.80
		Steroidobacterales	Steroidobacteraceae	Uncultured	3.44±0.82	0.00±0.00	0.22±0.19	2.25±0.25
		Xanthomonadales	Xanthomonadaceae	*Luteimonas*	1.53±0.27	0.26±0.02	0.96±0.74	0.76±0.29
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Sphaerochaeta*	1.96±0.63	0.00±0.00	0.16±0.08	0.41±0.19

Table S6. Relative abundance of taxa (%) significantly increased at DW sites compared to UP site over winter season. Significant difference between DW sites and UP site is indicated in bold ($p < 0.05$, DESeq2 test).

Phylum	Class	Order	Family	Genus	Creek sampling site			
					UP	WW	DW0	DW3000
Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae (Subgroup 1)	Uncultured	0.00±0.00	0.00±0.00	0.00±0.00	**0.22±0.17**
	Blastocatellia (Subgroup 4)	Blastocatellales	Blastocatellaceae	JGI 0001001-H03	0.09±0.05	0.00±0.00	0.05±0.06	**0.51±0.20**
				Uncultured	0.02±0.01	0.00±0.00	0.05±0.05	**0.51±0.30**
		DS-100	Uncultured bacterium	Unassigned	0.03±0.02	0.00±0.00	**0.24±0.15**	0.00±0.00
		Pyrinomonadales	Pyrinomonadaceae	RB41	0.05±0.02	0.00±0.00	**0.85±0.43**	0.07±0.09
	Holophagae	Holophagales	Holophagaceae	Marine group	0.06±0.02	0.00±0.00	**0.48±0.14**	**0.25±0.29**
		Subgroup 7	Uncultured bacterium	Uncultured	0.15±0.09	0.00±0.00	0.00±0.00	**0.58±0.41**
	Subgroup 6	Uncultured bacterium			1.35±0.31	0.00±0.00	0.11±0.10	**1.72±0.41**
		Unassigned	Unassigned	Unassigned	0.06±0.05	0.00±0.00	0.04±0.04	**0.66±0.44**
	Subgroup 18	Uncultured bacterium			0.05±0.02	0.00±0.00	0.00±0.01	**0.35±0.21**
	Subgroup 22	Uncultured bacterium			0.92±0.39	0.00±0.00	0.12±0.10	**1.96±1.01**
	Unassigned	Unassigned	Unassigned	Unassigned	0.07±0.04	0.00±0.00	0.01±0.01	**0.52±0.25**
Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.09±0.04	0.00±0.00	0.02±0.02	**0.24±0.16**
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.01±0.01	0.33±0.12	**0.91±1.06**	0.00±0.00
			Barnesiellaceae	Uncultured	0.16±0.09	0.05±0.03	**3.42±2.27**	0.02±0.04
			Paludibacteraceae	*Paludibacter*	0.03±0.04	0.01±0.01	**0.35±0.19**	0.00±0.00
			Prolixibacteraceae	*Roseimarinus*	0.63±0.29	1.07±0.26	**2.60±0.72**	0.30±0.10
			Rikenellaceae	Blvii 28 wastewater-sludge group	0.02±0.02	0.00±0.00	**0.54±0.39**	0.01±0.02
		Chitinophagales	Chitinophagaceae	*Ferruginibacter*	0.04±0.03	0.00±0.00	**0.15±0.21**	**0.23±0.11**
				Terrimonas	0.03±0.01	0.00±0.00	**0.23±0.32**	**0.51±0.08**
				Unassigned	0.10±0.03	0.00±0.00	0.17±0.22	**0.47±0.20**
			Saprospiraceae	*Haliscomenobacter*	0.10±0.06	0.00±0.00	**0.26±0.24**	**0.42±0.12**
				Saprospira	0.00±0.00	0.00±0.00	**0.16±0.16**	0.00±0.00
		Cytophagales	Microscillaceae	Uncultured	0.23±0.08	0.00±0.00	0.17±0.16	**0.49±0.17**
			Unassigned	Unassigned	0.03±0.02	0.06±0.00	**0.15±0.17**	**0.25±0.13**
		Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	1.29±0.31	21.67±1.27	**5.47±1.78**	0.47±0.09
				Unassigned	0.10±0.03	0.46±0.14	**0.35±0.11**	0.03±0.03
Calditrichaeota	Calditrichia	Calditrichales	Calditrichaceae	*Calorithrix*	0.00±0.00	0.00±0.00	0.00±0.00	**0.19±0.30**
Cloacimonetes	Cloacimonadia	Cloacimonadales	Uncultured bacterium		0.00±0.00	0.00±0.00	**0.30±0.33**	0.00±0.00
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Uncultured	0.23±0.11	0.00±0.00	0.05±0.04	**0.56±0.08**
		RBG-13-54-9	Uncultured bacterium		0.01±0.01	0.00±0.00	0.00±0.00	**0.39±0.49**
		SBR1031	A4b	Uncultured bacterium	0.10±0.01	0.00±0.00	0.06±0.08	**0.36±0.14**
			Uncultured bacterium		0.14±0.06	0.00±0.00	0.15±0.03	**1.19±0.51**
	KD4-96	Uncultured Chloroflexi bacterium			0.95±0.26	0.00±0.00	0.06±0.05	**1.15±0.51**
	Unassigned	Unassigned	Unassigned	Unassigned	0.03±0.03	0.00±0.00	0.00±0.01	**0.61±0.22**
Defferibacteres	Defferibacteres	Defferibacteriales	Defferibacteriaceae	Uncultured	0.12±0.03	0.12±0.07	**0.76±0.57**	0.03±.05
Epsilonbacteraeota	Campylobacteria	Campylobacteriales	Arcobacteraceae	*Arcobacter*	0.11±0.07	5.68±1.96	**2.36±1.07**	0.01±0.02
			Sulfurospirillaceae	*Sulfurospirillum*	0.01±0.01	0.03±0.01	**0.16±0.07**	0.00±0.00
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Carnobacterium*	0.02±0.02	0.05±0.04	**0.41±0.20**	0.00±0.00
	Clostridia	Clostridiales	Christesenellaceae	*Christesenellaceae* R7 Group	0.02±0.01	0.01±0.01	**0.42±0.22**	0.01±0.01
			Family XII	Uncultured	0.04±0.04	0.00±0.00	**0.29±0.13**	0.00±0.00
			Family XIII	Uncultured	0.05±0.03	0.00±0.00	**0.31±0.20**	0.01±0.02
			Lachnospiraceae	*Lachnoclostridium* 10	0.01±0.01	0.00±0.00	**0.17±0.19**	0.01±0.02
				Uncultured	0.02±0.01	0.15±0.04	**0.39±0.43**	0.00±0.00

			Ruminococcaceae	Unassigned	0.04±0.03	0.43±0.11	**0.27±0.16**	0.06±0.07
		Unassigned	Unassigned	Unassigned	0.17±.06	0.17±0.07	**0.30±0.13**	0.02±0.02
	Erysipelotrichia	Elysipelotrichiales	Erysipelotrichiaceae	*Solobacterium*	0.06±0.04	0.01±0.01	**0.75±0.15**	0.02±0.04
	Negativicutes	Selenomonadales	Acidaminococcaceae	Uncultured	0.00±0.00	0.09±0.03	**0.33±0.34**	0.00±0.00
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	0.03±0.02	0.00±0.00	0.01±0.01	**0.21±0.19**
				Uncultured	0.06±0.05	0.00±0.00	0.16±0.15	**1.81±0.78**
				Unassigned	0.01±0.01	0.00±0.00	0.07±0.08	**0.62±0.22**
Latescibacteria	Uncultured bacterium				0.26±0.11	0.00±0.00	0.15±0.12	**1.52±0.41**
	Uncultured Latescibacteria bacterium				0.00±0.01	0.00±0.00	0.02±0.03	**0.31±0.13**
	Unassigned	Unassigned	Unassigned	Unassigned	0.01±0.02	0.00±0.00	0.01±0.01	**0.34±0.16**
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	1.51±0.65	0.00±0.00	0.38±0.32	**3.53±2.41**
Proteobacteria	α-Proteobacteria	Caulobacterales	Hyphomonadaceae	UKL3-1	0.46±0.14	0.00±0.00	0.34±0.15	**1.41±0.54**
		Dongiales	Dongiaceae	*Dongia*	0.27±0.11	0.00±0.00	0.12±0.11	**0.86±0.34**
		Rhizobiales	A0389	Uncultured bacterium	0.00±0.01	0.00±0.00	0.02±0.02	**0.20±0.13**
			Xanthobacteraceae	Uncultured	0.35±0.19	0.00±0.00	0.05±0.06	**1.51±0.89**
		Sphingomonadales	Sphingomonadaceae	Unassigned	0.10±0.04	0.24±0.02	**0.67±0.31**	**0.53±0.15**
		Tistrellales	Tistrellaceae	*Tistrella*	0.04±0.04	0.00±0.00	0.04±0.04	**0.20±0.12**
		Uncultured	Uncultured Green bay ferromanganous micronodule bacterium MNH4		0.00±0.00	0.00±0.00	0.00±0.00	**0.22±0.12**
	δ-Proteobacteria	Desulfarculales	Desulfarculaceae	Uncultured	0.20±0.12	0.00±0.00	0.03±0.03	**0.32±0.17**
		Desulfobacterales	Desulfobacteraceae	SEEP-SRB1	0.26±0.06	0.00±0.00	0.02±0.02	**0.81±0.40**
			Desulfobulbaceae	*Desulfobulbus*	1.18±0.15	0.00±0.00	**1.56±0.78**	0.23±0.18
		Myxococcales	Phaselicystidaceae	*Phaselyscistis*	0.13±0.06	0.00±0.00	**0.24±0.20**	**0.58±0.24**
		MBNT15	Uncultured bacterium		0.05±0.01	0.00±0.00	0.01±0.01	**0.41±0.40**
		NB1-j	Uncultured bacterium		0.37±0.18	0.00±0.00	0.09±0.08	**1.37±0.72**
		Syntrophobacterales	Syntrophaceae	*Smithella*	0.42±0.14	0.00±0.00	0.07±0.03	**0.56±0.52**
		Unassigned	Unassigned	Unassigned	0.52±0.11	0.01±0.01	**1.72±1.40**	**2.92±0.43**
	γ-Proteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	0.13±0.07	9.89±0.77	**3.82±1.02**	0.01±0.03
		Alteromonadales	Pseudoalteromonadaceae	*Pseudoalteromonas*	0.00±0.00	0.32±0.20	**0.13±0.08**	0.00±0.00
		CCD24	metagenome		0.58±0.19	0.00±0.00	0.17±0.14	**0.94±0.29**
		Betaproteobacteriales	Burkholderiaceae	*Acidovorax*	0.17±0.06	9.61±1.33	**8.25±1.09**	0.25±0.13
				Caenimonas	0.01±0.01	0.00±0.00	0.04±0.03	**0.27±0.25**
				Massilia	0.05±0.01	0.64±0.04	**0.22±0.34**	**0.16±0.27**
				Polaromonas	0.03±0.01	0.05±0.02	**0.65±0.55**	0.00±0.00
				Rhodoferax	10.86±0.86	3.46±0.36	**13.33±2.35**	3.82±1.49
				Uncultured	2.32±0.57	2.49±0.28	0.69±0.56	**3.08±0.63**
			Gaiellaceae	*Sideroxydans*	0.20±0.10	0.07±0.02	**1.23±0.47**	**1.70±0.49**
			Hydrogenophilaceae	*Thiobacillus*	0.28±0.10	0.00±0.00	0.03±0.05	**0.41±0.43**
			Nitrosomonadaceae	Ellin6067	1.90±0.58	0.01±0.01	0.62±0.41	**3.97±0.51**
			Rhodocyclaceae	*Azoarcus*	0.04±0.02	0.01±0.01	**0.99±0.43**	**0.12±0.10**
				Thauera	0.00±0.00	0.00±0.00	**0.50±0.20**	0.00±0.00
				Unassigned	0.02±0.03	0.00±0.00	0.00±0.01	**0.39±0.73**
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.63±0.49	15.05±3.29	**3.00±0.78**	0.02±0.02
			Pseudomonadaceae	*Pseudomonas*	0.81±0.07	14.10±1.45	**4.94±1.04**	0.42±0.20
		Xanthomonadales	Xanthomonadaceae	*Luteimonas*	0.84±0.18	0.04±0.03	**1.96±1.34**	0.46±0.19
		Unassigned	Unassigned	Unassigned	2.79±1.05	0.35±0.05	2.20±0.21	**3.59±0.64**
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Spirochaeta 2*	0.02±0.01	0.00±0.00	0.02±0.03	**3.74±1.44**
				Treponema 2	0.20±0.10	0.01±0.01	**0.82±0.28**	0.07±0.66

				Unassigned	0.16±0.03	0.00±0.00	0.18±0.19	**0.73±0.03**
Synergistetes	Synergistia	Synergistales	Synergistaceae	*Aminomonas*	0.00±0.00	0.00±0.00	**0.47±0.3**	0.00±0.00

Sampling sites: UP, upstream of discharge, DW0, discharge site; DW3000, 3000 m downstream of discharge; WW - effluent.

Table S7. Relative abundance of taxa (%) significantly increased at DW sites compared to UP site over summer season. Significant difference between DW sites and UP site is indicated in bold ($p < 0.05$, DESeq2 test).

Phylum	Class	Order	Family	Genus	Sampling sites			
					UP	WW	DW0	DW3000
Acidobacteria	Blastocatellia (Subgroup 4)	Blastocatellales	Blastocatellaceae	Unassigned	0.04±0.04	0.00±0.00	0.05±0.03	**0.34±0.23**
	Holophagae	Holophagales	Holophagaceae	Marine group	0.15±0.14	0.04±0.01	**0.90±0.51**	0.09±0.04
		Subgroup 7	Uncultured bacterium SJA-36		2.05±0.67	0.00±0.00	**2.96±0.68**	0.68±0.19
	Subgroup 5	Uncultured Acidobacterium sp.			0.09±0.03	0.00±0.00	0.00±0.00	**0.15±0.08**
	Subgroup 6	metagenome			0.16±0.06	0.00±0.00	0.01±0.01	**0.26±0.04**
	Subgroup 18	Unassigned	Unassigned	Unassigned	0.03±0.03	0.00±0.00	0.00±0.00	**0.52±0.23**
	Subgroup 22	Uncultured bacterium			0.00±0.00	0.00±0.00	0.01±0.00	**0.36±0.18**
	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	Subgroup 10	0.19±0.14	0.00±0.00	0.00±0.00	**0.65±0.14**
	Unassigned	Unassigned	Unassigned	Unassigned	0.06±0.01	0.00±0.00	0.07±0.05	**0.67±0.11**
Actinobacteria	Coriobacteriia	OPB41	Uncultured bacterium		0.20±0.07	0.00±0.00	**0.33±0.08**	0.03±0.02
	Unassigned	Unassigned	Unassigned	Unassigned	0.19±0.04	0.00±0.00	**0.45±0.27**	**0.21±0.10**
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.00±0.01	4.06±0.14	**0.60±0.42**	0.00±0.00
			Bacteroidetes vadinHA17	Uncultured bacterium	0.00±0.00	0.00±0.00	**0.30±0.18**	0.01±0.01
			Barnesiellaceae	Uncultured	0.10±0.07	1.72±0.13	**2.45±0.58**	0.01±0.01
			Paludibacteraceae	*Paludibacter*	0.04±.03	0.01±0.01	**0.15±0.11**	0.01±0.01
			Prolixibacteraceae	*Roseimarinus*	0.28±0.15	1.99±0.04	**1.97±0.67**	0.29±0.09
			Rikenellaceae	Blvii28 wastewater-sludge group	0.03±0.03	0.00±0.00	**0.63±0.36**	0.01±0.02
				Uncultured	0.02±0.01	0.11±0.01	**0.17±0.11**	0.00±0.00
		Chitinophagales	Chitinophagaceae	*Terrimonas*	0.08±0.10	0.00±0.00	0.06±0.05	**0.47±0.05**
			Saprospiraceae	*Haliscomenobacter*	0.12±0.11	0.00±0.00	0.05±0.04	**0.30±0.14**
		Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	0.48±0.18	8.00±0.46	**0.66±0.18**	0.13±0.09
	Ignavibacteria	Kryptoniales	BSV26	Uncultured bacterium	0.00±0.00	0.00±0.00	0.00±0.00	**0.14±0.06**
Calditrichaeota	Calditrichia	Calditrichales	Calditrichaceae	*Calorithrix*	0.01±0.01	0.00±0.00	0.03±0.01	**0.44±0.30**
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Uncultured	0.20±0.05	0.00±0.00	0.04±0.02	**0.64±0.07**
		RBG-13-54-9	Uncultured bacterium		0.01±0.01	0.00±0.00	0.02±0.04	**0.56±0.39**
		SBR1031	A4b	Ambiguous taxa	0.03±0.05	0.00±0.00	0.00±0.01	**0.32±0.07**
				Uncultured bacterium	0.09±0.02	0.00±0.00	0.01±0.01	**0.47±0.05**
			Uncultured bacterium		0.41±0.13	0.00±0.00	**0.95±0.29**	**2.08±0.36**
	Dehalococcoidia	GIF9	Uncultured bacterium		0.00±0.00	0.00±0.00	0.02±0.01	**0.54±.25**
	Unassigned	Unassigned	Unassigned	Unassigned	0.01±0.01	0.00±0.00	0.01±0.01	**0.56±0.06**
Cloacimonetes	Cloacimonadia	Cloacimonadales	PBS-18	Uncultured bacterium	0.00±0.00	0.00±0.00	**0.18±0.16**	**0.22±0.12**
			Uncultured bacterium		0.00±0.00	0.00±0.00	**0.60±0.25**	0.00±0.00
			Unassigned	Unassigned	0.00±0.00	0.00±0.00	**0.27±0.12**	0.00±0.00
Epsilonbacteraeota	Campylobacteria	Campylobacterales	Arcobacteraceae	*Arcobacter*	0.02±0.02	14.28±0.60	**1.25±0.92**	0.02±0.02
			Thiovulaceae	*Sulfuricurvum*	0.22±0.13	0.06±0.01	**0.98±0.18**	0.07±0.02
Firmicutes	Bacilli	Bacillales	Bacillaceae	Unassigned	0.00±0.00	0.04±0.00	**0.22±0.09**	0.01±0.02
		Lactobacillales	Carnobacteriaceae	*Carnobacterium*	0.00±0.00	0.11±0.01	**0.14±0.07**	0.00±0.00
	Clostridia	Clostridiales	Christesenellaceae	*Christesenellaceae* R7 Group	0.06±0.05	0.04±0.01	**0.76±0.34**	0.01±0.01
			Family XIII	Uncultured	0.05±0.03	0.03±0.02	**0.39±0.08**	0.00±0.00
			Ruminococcaceae	Unassigned	0.14±0.03	0.13±0.04	**0.56±0.47**	0.06±0.05
			Unassigned	Unassigned	0.12±0.05	0.11±0.01	**0.44±0.19**	0.03±0.01
	Erysipelotrichia	Erysipelotrichiales	Erysipelotrichiaceae	*Solobacterium*	0.04±0.04	0.09±0.01	**1.21±0.61**	0.01±0.01
	Unassigned	Unassigned	Unassigned	Unassigned	0.26±0.04	0.07±0.02	**1.47±0.53**	0.07±0.02
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadaceae	Uncultured	0.31±0.36	0.00±0.00	0.13±0.13	**1.69±0.43**

				Unassigned	0.02±0.04	0.00±0.00	0.02±0.01	**0.55±0.14**
Latescibacteria	Latescibacteria	Latescibacteriales	Latescibacteriaceae	Uncultured bacterium	0.00±0.00	0.00±0.00	0.00±0.00	**0.25±0.17**
	Uncultured bacterium				0.30±0.17	0.00±0.00	**0.54±0.18**	**1.63±0.10**
	Unassigned	Unassigned	Unassigned	Unassigned	0.01±0.01	0.00±0.00	0.03±0.02	**0.31±0.05**
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	1.18±0.44	0.00±0.00	0.13±0.10	**3.59±1.67**
	Thermodesulfovibrionia	Uncultured	Uncultured Nitrospirae bacterium		0.00±0.00	0.00±0.00	0.00±0.00	**0.23±0.14**
	4-29-1	Uncultured bacterium			0.08±0.05	0.00±0.00	0.03±0.02	**0.50±0.07**
Proteobacteria	α-Proteobacteria	Caulobacterales	Hyphomonadaceae	UKL13-1	0.51±0.27	0.26±0.04	0.35±0.05	**1.53±0.07**
		Rhizobiales	A0839	Uncultured bacterium	0.03±0.05	0.00±0.00	0.00±0.00	**0.30±0.12**
			Beijerinckiaceae	*Psychroglaciecola*	0.00±0.00	0.10±0.03	**0.13±0.06**	0.04±0.04
			KF-JG30-B3	Metagenome	0.02±0.03	0.00±0.00	0.00±0.01	**0.24±0.11**
			Rhizobiales Inc. Sedis	Uncultured	0.03±0.03	0.00±0.00	0.01±0.01	**0.21±0.03**
			Xanthobacteraceae	Uncultured	0.27±0.24	0.00±0.00	0.08±0.03	**1.41±0.50**
			Unassigned	Unassigned	0.19±0.09	0.27±0.03	0.08±0.02	**0.62±0.06**
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	0.01±0.02	0.00±0.00	**0.23±0.10**	0.08±0.01
				Unassigned	0.04±0.07	0.13±0.01	**0.35±0.03**	**0.52±0.22**
		Unassigned	Unassigned	Unassigned	0.69±0.35	0.52±0.03	0.67±0.07	**1.82±0.54**
	δ-Proteobacteria	Desulfarculales	Desulfarculaceae	*Desulfatiglans*	0.01±0.01	0.00±0.00	0.01±0.00	**0.65±0.36**
		Desulfobacterales	Desulfobulbaceae	*Desulfobulbus*	1.40±0.33	0.00±0.00	**2.50±0.80**	0.18±0.08
				Desulfurivibrio	0.00±0.00	0.00±0.00	**0.80±1.36**	0.08±0.02
				MSBL7	0.00±0.00	0.00±0.00	**0.38±0.41**	0.01±0.01
				SEEP-SRB1	0.17±0.07	0.00±0.00	0.11±0.07	**0.72±0.16**
		Desulfuromonadales	Sva1033	Unassigned	0.00±0.00	0.00±0.00	**0.32±0.12**	0.00±0.00
			Unassigned	Unassigned	0.07±0.07	0.00±0.00	**0.24±0.25**	0.08±0.01
		Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*	0.00±0.00	0.02±0.02	**0.17±0.18**	0.00±0.01
			Phaselicystidaceae	*Phaselicystis*	0.35±0.15	0.00±0.00	0.37±0.31	**0.48±0.19**
		NB1-j	Uncultured bacterium		0.39±0.27	0.00±0.00	0.03±0.03	**1.20±0.24**
		Syntrophobacterales	Syntrophaceae	*Smithella*	0.45±0.14	0.00±0.00	**1.47±1.39**	**1.36±0.46**
			Syntrophobacteraceae	*Syntrophobacter*	0.03±0.02	0.00±0.00	**0.23±0.27**	0.05±0.02
		Unassigned	Unassigned	Unassigned	0.46±0.04	0.02±0.02	**1.84±0.47**	**2.66±0.50**
	γ-Proteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	0.13±0.03	9.71±1.25	**2.22±1.48**	0.02±0.03
			Succinivibrionaceae	Uncultured	0.01±0.01	0.02±0.00	**0.28±0.12**	0.00±0.00
		Alteromonadales	Shewanellaceae	*Shewanella*	0.00±0.00	0.19±0.03	**0.11±0.08**	0.00±0.00
		CCD24	metagenome		0.68±0.07	0.00±0.00	0.03±0.04	**1.09±0.18**
		Betaproteobacteriales	Burkholderiaceae	*Acidovorax*	0.11±0.02	13.30±0.24	**5.05±2.96**	**0.23±0.13**
				Uncultured	1.81±0.27	0.06±0.00	0.16±0.13	**3.51±0.65**
			Gallionellaceae	*Sideroxydans*	0.14±0.09	0.15±0.03	**0.65±0.28**	**1.53±0.53**
			Hydrogenophilaceae	*Thiobacillus*	0.42±0.01	0.00±0.00	0.05±0.04	**0.58±0.31**
			Nitrosomonadaceae	Ellin6067	1.98±0.22	0.00±0.01	0.66±0.20	**3.54±0.33**
			Rhodocyclaceae	*Azoarcus*	0.04±0.03	0.18±0.01	**1.00±0.43**	0.06±0.05
				Thauera	0.00±0.00	0.05±0.02	**1.24±0.45**	0.00±0.00
				Unassigned	0.05±0.02	0.02±0.00	0.00±0.00	**0.51±0.44**
			Unassigned		2.43±0.54	0.68±0.04	**2.68±0.72**	**3.43±1.51**
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.01±0.01	16.55±1.32	**1.97±1.35**	0.02±0.03
			Pseudomonadaceae	*Pseudomonas*	0.89±0.11	1.66±0.07	**1.20±0.81**	0.19±0.04
		Unassigned	Unassigned	Unassigned	2.92±0.31	4.51±0.13	**3.16±1.47**	**3.59±0.50**
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Spirochaeta 2*	0.08±0.04	0.00±0.00	**0.69±0.10**	**3.89±1.58**
				Treponema 2	0.48±0.13	0.01±0.01	**1.07±0.64**	0.09±0.01
				Uncultured	0.96±0.43	0.00±0.00	**7.38±2.07**	**1.24±0.23**
				Unassigned	0.28±0.12	0.00±0.00	**0.37±0.17**	**0.71±0.22**

Synergistetes	Synergistia	Synergistales	Synergistaceae	*Aminomonas*	0.00±0.00	0.02±0.01	**0.57±0.06**	0.00±0.00

Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge; WW - effluent.

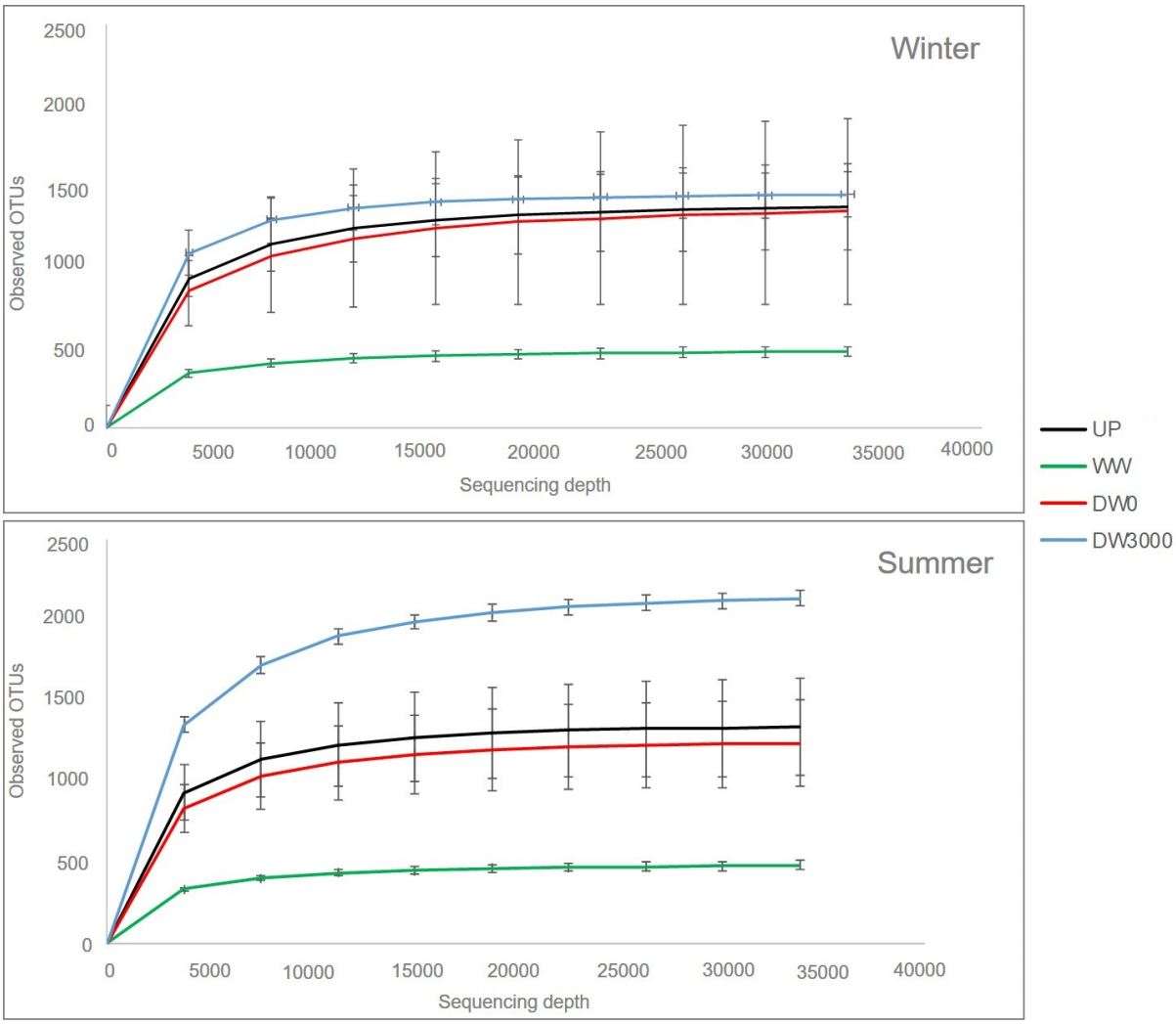

Figure S1. Rarefaction curves showing alpha diversity of drug-formulation effluent (WW) and sediments samples of the receiving creek using metrics of the observed species (i.e. ASVs). Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

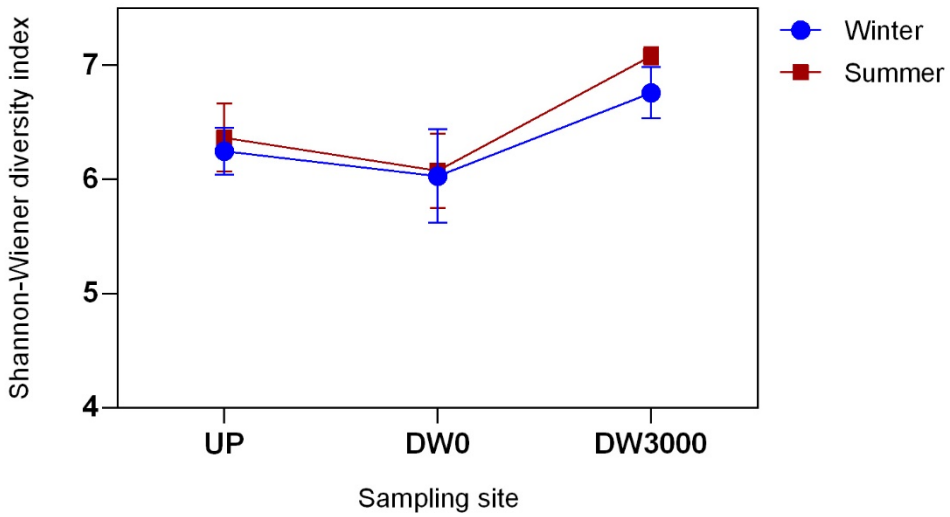

Figure S2. Shannon-Wiener diversity index of sediments taken from the creek receiving effluents from drug-formulation industry. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

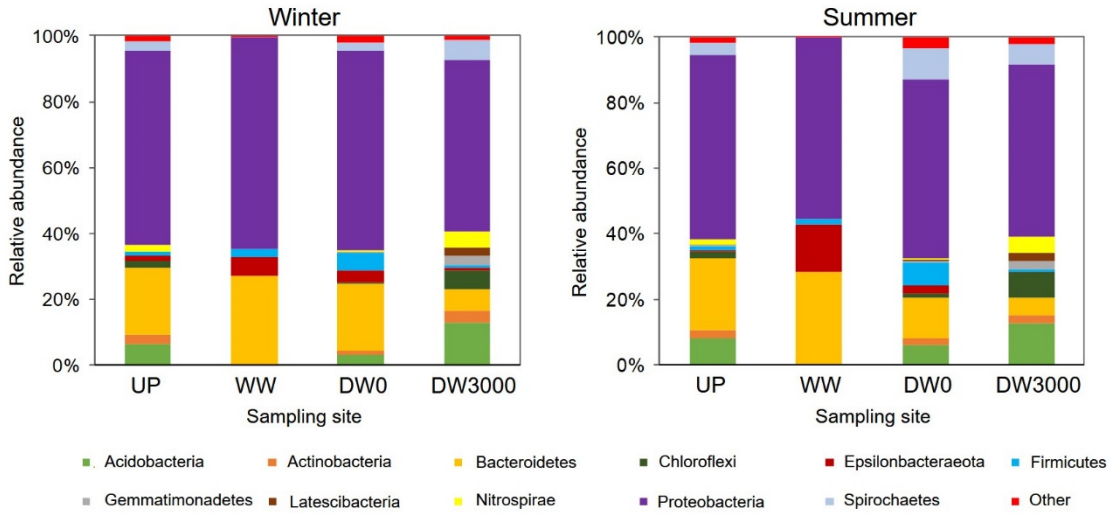

Figure S3. Taxonomic composition of bacterial communities at phylum level in samples of effluent (WW) and creek sediments receiving effluents from formulation facility over winter and summer season. Taxa with abundance below 1% in all samples were group into 'Other'. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

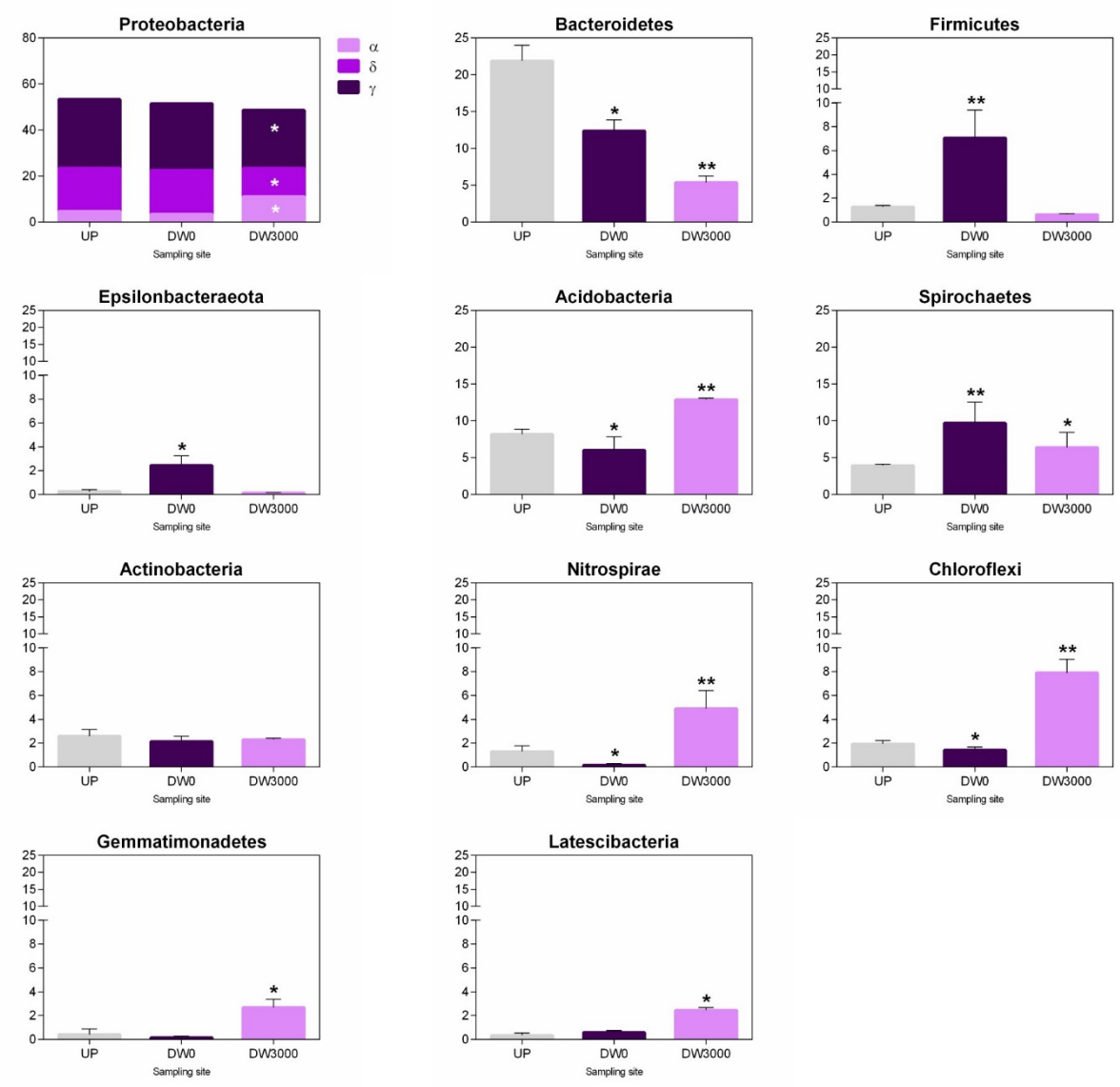

Figure S4. Changes in bacterial community composition at phylum level in sediments from different sites along the creek over summer season. The relative abundance of phyla is shown based on their closest match to SILVA database at 99% similarity level. Asterisks represents a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; DESeq2) between each DW site and UP site. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.