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

Milena Milaković, Gisle Vestergaard, Juan Jose González-Plaza, Ines Petrić, Josipa Kosić-Vukšić, Ivan Senta, Susanne Kublik, Michael Schloter, Nikolina Udiković-Kolić

PII: S0048-9697(19)35997-2

DOI: https://doi.org/10.1016/j.scitotenv.2019.136001

Reference: STOTEN 136001

To appear in: *Science of the Total Environment*

Received date: 30 September 2019

Revised date: 21 November 2019

Accepted date: 6 December 2019

Please cite this article as: M. Milaković, G. Vestergaard, J.J. González-Plaza, et al., 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, *Science of the Total Environment* (2019), https://doi.org/10.1016/j.scitotenv.2019.136001

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

Milena Milaković[a], Gisle Vestergaard[b,c], Juan Jose González-Plaza[a1], Ines Petrić[a], Josipa Kosić-Vukšić[d], Ivan Senta[a], Susanne Kublik[b], Michael Schloter[b], Nikolina Udiković-Kolić[a,*]

[a] Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička 54, P.O. Box 180; 10 002 Zagreb, Croatia

[b] Research Unit for Comparative Microbiome Analysis (COMI), Helmholtz Zentrum München, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany

[c] Section for bioinformatics, Department of Health Technology, Technical University of Denmark, DK-2800 Lyngby, Denmark

[d] Andrija Štampar Teaching Institute of Public Health, Mirogojska cesta 16, 10 000 Zagreb, Croatia

* Corresponding autor:
Dr. Nikolina Udiković-Kolić
Division for Marine and Environmental Research
Ruđer Bošković Institute
Bijenička 54, P.O. Box 180; 10 002 Zagreb, Croatia
E-mail address: nudikov@irb.hr

[1] Present address: Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Kamýcká 129, Prague 6 Suchdol, Czech Republic

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 of less antibiotic-polluted effluents (µg/L) from drug-formulation on exposed aquatic microbial communities 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 5.08 mg/kg) and azithromycin (up to 0.39 mg/kg) at DW0, but sulfonamides accumulated at DW3000 (total up to 1.17 mg/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 UP site. 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), 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; Lübbert et al., 2017; Šimatović and Udiković-Kolić, 2019). This increase in ARG abundance 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 selective pressure from antibiotics (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 pathogenic bacteria. 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; Sidrach-Cardona et al., 2014). 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). Despite these detrimental environmental effects, there are still no established limits for releases of antibiotics from companies producing and formulating antibiotics nor for the content of antibiotics in the environment.

In contrast to such high antibiotic loads in effluents from antibiotic production companies, effluents from companies involved in the formulation of drugs contains 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 (<10 µg/L) (Michael et al., 2013; Zhou et al., 2019), which were also shown to increase the abundance, diversity and potential spread of ARGs in recipient water bodies (Osinska et al., 2017; Lekunberri et al., 2018; 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 trimethoprim, in concentrations up to approximately 250 µg/L. More recently (González-Plaza et al., 2018, 2019), we also demonstrated that these effluents were sources of diverse ARGs 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, sample collection and DNA extraction

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, including antibiotics mainly from sulfonamide, tetracycline, ß-lactam, diaminopyridine and macrolide classes.

Sediment samples were collected from 3 sites along the recipient 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 included reference site (300 m upstream, UP), effluent discharge site (DW0) and one site downstream of the discharge (3000 m downstream, DW3000) (González-Plaza et al., 2019). The sites UP and DW0 are located in agricultural area, while site DW3000 is located in forest area where no agriculture is taking place and the flow rate is slower than at DW0 site. 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 and immediately transported to the laboratory on ice. 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 wastewater samples of the industry as described recently (Bielen et al., 2017; González-Plaza et al., 2018, 2019).

Aliquots of wastewater samples (50-100 mL), collected in two sampling campaigns (winter and summer), were vacuum-filtered through a 0.22 µm pore-size membrane (GE Healthcare Life Sciences, PA, USA) and filters were stored at -80°C until DNA extraction. DNA was extracted from filters and sediment cores using the Power Soil DNA isolation kit (MoBio, CA, USA.) Non-template extraction control was set up using DNA-free water. Extracted DNA was stored at -20°C until use.

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 ISO standardized methods (Milaković et al., 2019). Quantification of antibiotics (sulfadiazine, SDZ; sulfamethazine, SMZ; trimethoprim, TMP; and azithromycin, AZI) in sediments was performed following the analytical methodology and protocols previously described (Senta et al., 2008, 2013). The contents of heavy metals (Cd, Cr, Cu, Pb, Ni, Zn, K, Na, Li, Mg, Fe, Mn, Cs, Rb, Al, Sr, Ba, Be) were measured by inductively coupled plasma mass spectrometry as described previously (Dautović et al., 2014).

2.3. Quantification of ARGs and 16S rRNA genes

ARGs conferring resistance to tetracyclines (*tet*C and *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) were quantified in effluent and sediment samples using SYBR-Green quantitative real-time PCR (qPCR). The 16S rRNA gene (*rrn*) was also analyzed for normalization of the data. All qPCR assays were performed using ABI 7300 Real-time PCR system (Applied Biosystems, CA, USA) and the reaction setup as described

previously (Milaković et al., 2019). Specific primer sets, annealing temperatures, amplification accuracies and efficiencies are listed in Table S1. Standard curves were prepared by cloning target genes (amplified using gene-positive bacteria) into JM109 competent cells using a pGEM-T Easy vector cloning kit (Promega, France) (Milaković et al., 2019). Efficiency and accuracy values (Table S1) were determined from six points of the serial dilutions of plasmid carrying ARG. For all but three ARGs (i.e. $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$, and *erm*B) the following program was used: 95 °C for 15 min, 30 cycles at 95 °C for 15 s, annealing at corresponding temperature (Table S1) for 30 s, and 72 °C for 30 s. A previously described qPCR conditions were used for quantification of $bla_{\text{OXA-1}}$ and $bla_{\text{OXA-2}}$ (Zhai et al., 2016), *rrn (*López-Gutiérrez et al., 2004) and *erm*B (Chen et al., 2007). The quantification limit for all target ARGs was 10^2 gene copies per reaction. To minimize the variance in bacterial concentration or amplification efficiency between samples, the relative gene abundance was expressed as the ratio of ARG copy number per 16S rRNA gene copy number.

2.4. Bacterial community analyses

The bacterial community composition was analyzed by 16S rRNA gene amplicon sequencing as described previously (Milaković et al., 2019). Briefly, amplicon libraries of the V1-V2 hypervariable region of the bacterial 16S rRNA gene were prepared from each extracted DNA sample and barcode sequenced using the Miseq platform (Illumina, United Kingdom, Chesterford). The QIIME 2 v2018.2.0 (https://qiime2.org) was used for bioinformatic analysis, and amplicon sequencing variants (ASVs) were taxonomically classified using the SILVA v132 database. Alpha diversity was estimated based on calculated Shannon diversity indices and generated rarefaction curves. Beta-diversity between samples

was examined using Bray-Curtis dissimilarity and ordinated using non-metric multidimensional scalling (NMDS).

2.5. Statistical analyses

All statistical analyses, except Kruskal-Wallis test, were performed in R studio (v1.1.383). Shapiro Wilk's test was performed to test for normal distribution of $\log_{10}$ transformed qPCR data with "fitdistrplus" and "stats" packages. Kruskal-Wallis test (performed in GraphPad Prism v6.01) and the package DESeq2 (v1.22.1) were used to test statistically significant differences ($p < 0.05$) of the relative abundance of ARGs and bacterial phyla/genera between each DW site and UP site (Milaković et al., 2019). Co-occurrence patterns of ARGs and the bacterial genera were revealed by a network analysis (Li et al., 2015), based on the Spearman's correlation. Networks were visualized using Cytoscape v3.7.0. (Shannon et al., 2003). A correlation between two nodes was statistically significant if $\rho > 0.7$ and the p-value was < 0.01. To avoid false-positive correlations, the p-values were adjusted by using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

3. RESULTS

3.1. Physical and chemical properties of sediments

As summarized in Table S2, the sediment samples were slightly acidic to alkaline (pH 6.81–7.98) with a silty-sand texture (silt 53 –67%, sand 27 –43%) (Wenthworth, 1922). The sediments from DW0 site had the lowest TOC (max 2.13%), TC (max 3.34%) and TN (max 0.17%) values over both seasons. In contrast, the maximum value of NO_3^- (24 mg/kg) was observed at this site during summer, and NH_4^+ (16 mg/kg) during winter.

All target antibiotics (sulfadiazine, sulfamethazine, trimethoprim and azithromycin) were detected in sediments from all sampling sites, being present in the lowest levels (≤ 0.04 mg/kg) at UP during both seasons (Table 1). In contrast, the highest levels of TMP (up to 5.08 mg/kg) and AZI (up to 0.39 mg/kg) were detected at DW0 during both seasons, 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 amounts at DW3000 compared to UP. For SDZ and SMZ, the highest amounts were not found at site DW0 but at site DW3000 (total 1.17 mg/kg - winter and 0.56 mg/kg - summer, Table 1).

For the analysis of heavy metals, Cd, Cr, Cu, Pb, Ni, and Zn were chosen as primary targets because they have previously been reported to promote antibiotic resistance (Seiler and Berendonk, 2012). The concentration of Cr, Pb and Ni was slightly higher at DW0 compared to UP only during winter (Table S3). Surprisingly, highest concentrations of both Cu and Zn were measured in sediments from UP during both seasons, especially of Zn (475 mg/kg two-season average), 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, i.e. concentration needed to co-select for metal and antibiotic resistance (Seiler and Berendonk, 2012). In addition to the above-mentioned metals, we also measured other metals not associated with antibiotic resistance, such as K, Na, Li, Mg, Fe, Mn, Cs, Rb, Al, Sr, Ba, Be, and their concentrations were generally lower in DW sediments compared with UP or equal among all sampling sites (data not shown).

3.2. 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 were *sul*1, *sul*2, *mph*G, *msr*E, *tet*C, *tet*39, *dfr*A14 and bla_{OXA-2}, with an average values of the two seasons mainly in the range of -1 to -2 log gene copies/*rrn* copies (Table S4; Fig. 1). However, the relative abundances of bla_{GES}, bla_{VEB}, bla_{OXA-1}, *mef*C and *erm*B were in most cases 10-times lower (approximately -3 log units), while the relative abundance of *fol*A and *mph*E subtypes was approximately 100-times lower (around -4 log units) (Table S4).

In creek sediments at UP site, the *sul*1*, dfrA*14, *tet*C and bla_{VEB} genes were detected at relatively high abundances of approximately -2 log units (two-season average), while the relative abundances for *tet*39, bla_{OXA-1}, bla_{OXA-2}, *sul*2 and *fol*A were around -3 log units (Fig.1, Table S4). Genes *mph*G and *msr*E were detected only during winter (around -4 log units) while *mph*E and *mef*C were detected only during summer (around -2.5 log units). In contrast, the genes bla_{GES} and *erm*B were below quantification limit in UP sediment in both seasons (Table S4).

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 S4). Also, seasonal differences in the relative abundance of ARG subtypes were observed. Specifically, among target ß-lactam ARGs, relative abundances of both bla_{GES} and bla_{OXA-1} subtypes increased by more than one order of magnitude at DW0 during both seasons, while bla_{OXA-2} subtype was

about 0.8 log units higher at DW0 compared to UP only during summer (Fig. 1 and Table S4). Regarding TMP resistance, only the difference in abundance of *dfr*A14 between DW0 and UP was significantly increased (about 0.8 log units) during summer. Considering macrolide ARGs, *erm*B subtype significantly increased by more than two orders of magnitude at DW0 compared to UP during both seasons, while *mph*E and *mef*C were found elevated only in winter (Fig. 1 and Table S4). During summer, *mph*G and *msr*E subtypes significantly increased in relative abundance (up to three orders of magnitude) not only at DW0, but also at DW3000 compared to UP ($p < 0.05$; Kruskal-Wallis; Fig. 1 and Table S4). Similar to this, the sulfonamide resistance gene *sul*2 increased in relative abundance by around one order of magnitude at both DW0 and DW3000 compared to UP in both seasons, whereas *sul*1 increased only at DW0 compared to UP. In contrast, tetracycline ARGs were found significantly elevated during summer only at DW0 site, with increases of around one order of magnitude (Fig. 1, Table S4).

3.3. Impact of formulation effluents on sediment bacterial communities

A total of 2,265,504 high-quality reads from effluent and sediment samples were obtained after quality-filtering. Those were assigned to a 13,461 ASVs at 99% similarity level, which were used for all downstream analyses. 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).

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 during both seasons, as indicated by Shannon-Wiener diversity index (Fig. S2). However, the structure of sediment bacterial communities was found to be

significantly altered according to NMDS analysis based on Bray-Curtis dissimilarity. Sediment samples from three studied sites (UP, DW0 and DW3000) clustered separately (Adonis R^2 = 0.8254, $p < 0.05$), independent from sampling season (Fig. 2).

At the phylum level, *Proteobacteria* and *Bacteroidetes* were the most abundant in all sediment and effluent 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, *Spirochaetes* and *Chloroflexi* dominated in DW3000 sediments (Fig. S3). Effluent discharge resulted in significant changes ($p < 0.05$) in the relative abundance of different phyla at DW0 and DW3000 compared to UP site as shown by DESeq2 analysis (Fig. S4). Phyla such as *Firmicutes* and *Epsilonbacteraeota* showed the most significant increase in the relative abundance at DW0 compared to UP during both seasons (increased 5.2% and 2%, respectively; two-season average), but decreased to background levels (*Firmicutes*) or significantly below background levels (*Epsilonbacteraeota*) at DW3000. Several phyla significantly increased at DW3000 versus UP over both seasons, including *Acidobacteria* (5.6% two-season average), *α-Proteobacteria* (5.4%), *Chloroflexi* (4.9%), *Nitrospirae* (3.4%), *Spirochaetes* (2.9%), *Gemmatimonadetes* (2.6%) and *Latescibacteria* (2.1%) (Fig. S4).

At the family or genus level, some bacteria that were highly abundant (>1%) in sediments from UP site in both seasons, such as *Prolixibacteraceae* (BSV13, *Prolixibacter*, WCHB1-32), *Cyclobacteraceae*, *Ignavibacterium, Geobacter, Anaeromyxobacter, Steroidobacteraceae* and *Sphaerochaeta*, significantly decreased in sediments from both DW sites (Tables S5 and S6). In contrast, genera that were highly abundant (≥1%) in effluents in both seasons, such as *Acidovorax, Aeromonas, Pseudomonas*, *Acinetobacter*,

Flavobacterium, *Roseimarinus* and *Arcobacter* (Fig. 3), significantly increased in relative abundance in sediments from DW0 compared to UP, but not at DW3000 in both seasons (Fig. 3, Tables S7 and S8). Besides, various other genera with low abundance in effluents (<0.1%) and UP sediment (≤0.8%) also showed significantly increased abundance in DW0 (>1%) compared to UP, with differences between seasons. These, for instance, included *Sideroxydans* (1.2%) and *Luteimonas* (1.9%) in winter, and *Solobacterium* (1.2%), *Treponema*2 (1%) and *Smithella* (1.4%) in summer (Fig. 3, Tables S7 and S8). In addition, some psychrophilic genera such as *Sulfurospirillum, Pseudoalteromonas*, *Massilia*, and *Polaromonas* increased in abundance at DW0 compared to UP only in winter, but their proportion was generally low (≤0.6%). Further, some genera were significantly increased in relative abundance at DW0 in both seasons, including those specific to sediment (*Desulfobulbus* – 2%) or effluent (*Thauera* – 0.9%). 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. 3, Tables S7 and S8). In addition, unassigned members of the family *Sphingomonadaceae* were found in significantly increased relative abundance at both DW0 and DW3000 sites compared to UP during both seasons. There were few other taxa 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. 3, Tables S7 and S8). 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. 3, Tables S7 and S8). Notably, many of the taxonomic groups

with significantly higher abundances at DW3000 compared to UP could not be classified to the genus level.

3.4. 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. 4). 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 of them (bla_{GES}, bla_{OXA-1}, bla_{OXA-2}, *sul*1, *sul*2, *tet*39, *tet*C, *dfr*A14, *mp*hG, *msr*E, and *erm*B) were significantly positively correlated with bacterial genera. In total, 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. 4). 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. 4, Tables S7 and S8). 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. 4, Tables S7 and S8). 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. 4, Tables S8).

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 DW0 site for both trimethoprim (up to 5.08 mg/kg) and azithromycin (up to 0.39 mg/kg), whereas the total concentration of two sulfonamides was the highest at the site located 3 km downstream (up to 1.17 mg/kg). The latter could be the consequence of the slower flow rate of the creek at DW3000 compared to DW0, which might accelerate the deposition of antibiotics into the sediment. 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 0.6 mg/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 0.04 mg/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 (≥ -2.5 log gene copies/*rrn*) of the ARGs conferring resistance to sulfonamides (*sul*1, *sul*2), tetracyclines (*tet*39, *tet*C), macrolides (*mph*G, *msr*E), ß-lactams ($bla_{\text{OXA-2}}$), 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

ARGs, despite the relatively high ARGs abundance already present in the background sediments (UP site). In addition to the above-mentioned highly abundant ARGs, effluents also contained moderate amounts (on average -3 to -4 log ARG copies/*rrn*) of various ARG subtypes encoding resistance to ß-lactams (bla_{GES}, bla_{VEB}, bla_{OXA-1}), trimethoprim (*fol*A) and macrolides (*erm*B, *mph*E, *mef*C). Most of these ARGs showed different dynamics during winter and summer sampling, but were always higher in relative abundance at DW0 compared to UP, with the exception of *fol*A, *erm*B and bla_{GES}. These differences between seasons may be explained by variations in background sediment levels of analyzed ARGs.

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

However, most of ARGs with increased abundance in sediments at DW0 did dissipate to background levels at the more distant DW3000 site. These results indicate either limited transport / death of bacterial hosts (Milaković et al., 2019), degradation of extracellular DNA containing ARGs (Nnadzoie and Odume, 2019), binding of ARGs to sediment (Calero-Cáceres

et al., 2017), or a combination. Nevertheless, three gene subtypes, i.e. *sul*2, *mph*G and *msr*E, were detected significantly elevated above background also at DW3000 site. This might be due to growth of their hosts as a result of selection pressure from residual antibiotics (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. In contrast, the hypothesis 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 0.15 mg/kg (Heuer et al., 2008), which is lower than 0.16 mg SDZ/kg (summer) and 0.69 mg 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 (0.35 mg/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 DW sediment samples, suggesting that a wide range of bacteria are able to survive at high concentrations of antibiotic mixtures. A similar conclusions was reached in other studies investigating community changes in response to high antibiotic selection pressure (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 NMDS 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 that were more abundant in sediments than in effluents, such as *Acidobacteria*, *Chloroflexi, Nitrospirae, Gemmatimonadetes, Latescibacteria* and *α-Proteobacteria,* 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., 2011) and from urban WWTP (Piotrowska et al., 2017), suggesting that waterborne *Aeromonas* species can be important reservoirs and vehicles for dissemination of extended-spectrum ß-lactamases in the environment (Harnisz and Korzeniewska, 2018). Some taxa took along 3-4 ARGs including *Arcobacter* (bla_{GES}, *tet*39, *erm*B), *Acinetobacter* (bla_{GES}, bla_{OXA-2}, *tet*39) or *Shewanella* (bla_{GES}, bla_{OXA-1}, *tet*39, *erm*B). The last three genera together with *Aeromonas* had been considered as the opportunistic human and/or animal pathogenic bacteria (Janda and Abbott, 2010; Janda, 2014; Ferreira et al., 2015; Wong et al., 2017), suggesting that industrial effluent discharge increased the prevalence of pathogenic bacteria carrying multiple ARGs of clinical relevance in the receiving creek sediments. This may increase the risk of direct transmission of these multi-resistant pathogens to humans. However, for ARGs with higher prevalence at DW3000 versus UP site (*sul*2, *mph*G and *msr*E), we found an association of only *sul*2 with ASVs of the family *Sphingomonadaceae* which were more abundant at both DW sites than at UP site. This family has already been linked with sulfonamide resistance (Narciso da Rocha et al., 2014; Vaz-Moreira et al., 2011) and assumed for being prone for acquiring *sul* genes (Narciso da Rocha et al., 2014).

5. CONCLUSIONS

The present study revealed that effluent discharges from local drug-formulation facility contributed to pollution of the receiving creek sediments with antibiotics and ARGs despite relatively high background levels of the investigated genes in the creek. In addition, effluent discharge caused pronounced changes in sediment bacterial communities from both

downstream sites compared to upstream, but the overall taxonomic diversity was not affected. In contrast to effluent discharge site where increased levels of analyzed ARGs are likely a consequence of deposition of effluent-associated bacteria, the accumulated levels of sulfonamides at more distant downstream site could play a role in shifting community composition and increasing some sulfonamide and macrolide ARGs. Our results demonstrate the necessity for implementing/improving infrastructure for the treatment of sewage and industrial waste in the analyzed region in order to limit environmental transmission of antibiotic residues and antibiotic resistance determinants.

Data accesibility

The 16S rRNA gene sequences that support the findings of this study have been deposited in GenBank within the BioProject with the accession code PRJNA588393.

Acknowledgments

This work has been supported by the Croatian Science Foundation under the project number UIP-2014-09-9350. Dr. Gisle Vestergaard was supported by a Humboldt Research Fellowship for postdoctoral researchers. We thank Dr. Ana Bielen and Dr. Ana Šimatović for assistance in sample collection and processing, and to Nenad Muhin for the tehnical assistance.

References

Baena-Nogueras, R.M., González-Mazo, E., Lara-Martín, P.A., 2017. Degradation kinetics of pharmaceuticals and personal care products in surface waters: photolysis vs biodegradation. Sci. Total Environ. 590-591, 643-654. https://doi.org/10.1016/j.scitotenv.2017.03.015

Bengtsson-Palme, J., Milakovic, M., Švecová, H., Ganjto, M., Jonsson, V., Grabic, R., Udikovic-

Kolic, N., 2019. Industrial wastewater treatment plant enriches antibiotic resistance genes and alters the structure of microbial communities. Wat. Res. 162, 437-445. https://doi.org/10.1016/j.watres.2019.06.073

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bielen, A., Šimatović, A., Kosić-Vukšić, J., Senta, I., Ahel, M., Babić, S., Jurina, T., González Plaza, J.J., Milaković, M., Udiković-Kolić, N., 2017. Negative environmental impacts of antibiotic-contaminated effluents from pharmaceutical industries. Water Res. 126. https://doi.org/10.1016/j.watres.2017.09.019

Calero-Cáceres, W., Méndez, J., Martín-Díaz, J., Muniesa, M., 2017. The occurrence of antibiotic resistance genes in a Mediterranean river and their persistence in the riverbed sediment. Environ. Poll. 223, 384-394. https://doi.org/10.1016/j.envpol.2017.01.035

Carlsson, O., Porcher, J.M., Sanchez, W., 2009. Effluent from bulk drug production is toxic to aquatic vertebrates. Environ. Toxicol. Chem. 28, 2656-2662. https://doi.org/10.1897/08-524.1

Chen, J., Yu, Z., Michael, F.C., Wittum, T., Morisson, M., 2007. Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. Appl. Environ. Microbiol. 73, 4407-4416. https://doi.org/10.1128/AEM.02799-06

Corno, G., Yang, Y., Eckert, E.M., Fontaneto, D., Fiorentino, A., Galafassi, S., Zhang, T., Di Cesare, A., 2019. Effluents of wastewater treatment plants promote the rapid stabilization of the antibiotic resistome in receiving freshwater bodies. Wat. Res. 158, 72-81. https://doi.org/10.1016/j.watres.2019.04.031

Dautović, J., Fiket, Ž., Barešić, J., Ahel, M., Mikac, N., 2014. Sources, distribution and behavior of major and trace elements in a complex karst lake system. Aquat. Geochem. 20, 19–38. https://doi.org/10.1007/s10498-013-9204-9

Ferreira, S., Queiroz, J., Oleastro, M., Domingues, F.C., 2015. Insights in the pathogenesis and resistance of *Arcobacter*: a review. Crit. Rev. Microbiol. 42, 364-383. https://doi.org/10.3109/1040841X.2014.954523

Flach, C.F., Johnning, A., Nilsson, I., Smalla, K., Kristiansson, E., Larsson, D.G.J., 2015. Isolation of novel IncA/C and IncN fluoroquinolone resistance plasmids from an antibiotic-polluted lake. J. Antimicrob. Chemother. 70, 2709–2717. https://doi.org/10.1093/jac/dkv167

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science. 337, 1107-1111. https://doi.org/10.1126/science.1220761

Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistome across habitats. Nature. 509, 612-616. https://doi.org/10.1038/nature13377

Gatica, J., Tripathi, V., Green, S., Manaia, C.M., Berendonk, T., Cacace, D., Merlin, C., Kreuzinger, N., Schwartz, T., Fatta-Kassinos, D., Rizzo, L., Schwermer, C.U., Garerlick,

H., Jurkevitch, E., Cytryn, E., 2016. High througput analysis of integron gene cassettes in wastewater environments. Environ. Sci. Technol. 50, 11825-11836. https://doi.org/10.1021/acs.est.6b03188

Girlich, D., Poirel, L., Nordmann, P., 2011. Diversity of clavulanic acid-inhibited extended-spectrum β-lactamases in *Aeromonas* spp. from the Seine river, Paris, France. Antimicrob. Agents Chemoter. 55, 1256-1261. https://doi.org/10.1128/AAC.00921-10

González-Plaza, J.J., Blau, K., Milaković, M., Jurina, T., Smalla, K., Udiković-Kolić, N., 2019. Antibiotic-manufacturing sites are hot-spots for the release and spread of antibiotic resistance genes and mobile genetic elements in receiving aquatic environments. Environ. Int., 104735. https://doi.org/10.1016/j.envint.2019.04.007

González-Plaza, J.J., Šimatović, A., Milaković, M., Bielen, A., Wichmann, F., Udiković-Kolić, N., 2018. Functional repertoire of antibiotic resistance genes in antibiotic manufacturing effluents and receiving freshwater sediments. Front. Microbiol. 8, 1-13. https://doi.org/10.3389/fmicb.2017.02675

Gothwal, R., Shashidhar, 2017. Occurrence of high levels of fluoroquinolones in aquatic environment due to effluent discharges from bulk drug manufacturers. J. Hazard. Toxic Radioact. Waste. 21, 05016003. https://doi.org/10.1061/(ASCE)HZ.2153-5515.0000346

Guang, Y., Jia, J., Wu, L., Xue, X., Zhang, G., Wang, Z., 2019. Analysis of bacterial community characteristics, abundance of antibiotics and antibiotic resistance genes along a pollution gradient of Ba river in Xi'an, China. Front. Microbiol. 9, 1-12. https://doi.org/10.3389/fmicb.2018.03191

Guo, Y., Deng, X., Liang, Y., Zhang, L., Zhao, G.-P., Zhou, Y., 2018. The draft genomes and

investigation of serotype distribution, antimicrobial resistance of group B *Streptococcus* strains isolated from urine in Suzhou, China. Ann. Clin. Microbiol. Antimicrob. 17, 1-7. https://doi.org/10.1186/s12941-018-0280-y

Halliday, E., McLellan, S.L., Amaral-Zettler, L.A., Sogin, M.L., Gast, R.J., 2014. Comparison of bacterial communities in sands and water at beaches with bacterial water quality violations. PLOS One. 9, 1-9. https://doi.org/10.1371/journal.pone.0090815

Harnisz, M., Korzeniewska, E., 2017. The prevalence of multidrug-resistant *Aeromonas* spp. in the municipal wastewater system and their dissemination in the environment. Sci. Total Environ. 262, 377-383. https://doi.org/10.1016/j.scitotenv.2018.01.100

Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9, 657-666. https://doi.org/10.1111/j.1462-2920.2006.01185.x

Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., Spiteller, M., 2008. Fate of sulfadiazine administred to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biol. Biochem. 40, 1892-1900. https://doi.org/10.1016/j.soilbio.2008.03.014

Ibekwe, A.M., Ma, J., Murinda, S.E., 2016. Bacterial community composition and structure in an urban river impacted by different pollutant sources. Sci. Total Environ. 566, 1176-1185. https://doi.org/10.1016/j.scitotenv.2016.05.168

Janda, J.M., 2014. *Shewanella*: a marine pathogen as an emerging cause of human disease. Clin. Microbiol. Newsl. 36, 25-29. https://doi.org/10.1016/j.clinmicnews.2014.01.006

Janda, J.M., Abbott, S.L., 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection.

Clin. Microbiol. Rev. 23, 35-73. https://doi.org/10.1128/CMR.00039-09

Khan, G.A., Berglund, B., Khan, K.M., Lindgren, P.E., Fick, J., 2013. Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities - a study in Pakistan. PLOS One. 8, 1-8. https://doi.org/10.1371/journal.pone.0062712

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H., Larsson, D.G.J., Rodriguez-Valera, F., Miguel Hernandez, U., 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS One. 6, 1-7. https://doi.org/10.1371/journal.pone.0017038

Larsson, D.G.J., 2014. Pollution from drug-manufacturing: review and perspectives. Phil. Trans. R. Soc. B 369, 1-7. https://doi.org/10.1098/rstb.2013.0571

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., Qi, R., 2010. Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. Appl. Environ. Microbiol. 76, 3444-3451. https://doi.org/10.1128/AEM.02964-09

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J.M., Zhang, T., 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. 9, 2490–2502. https://doi.org/10.1038/ismej.2015.59

Li, S., Zhang, R., Hu, J., Shi, W., Kuang, Y., Guo, X., Sun, W., 2019. Occurrence and removal of antibiotics and antibiotic resistance genes in natural and constructed riverine wetlands in Beijing, China. Sci. Total Environ. 664, 546-553. https://doi.org/10.1016/j.scitotenv.2019.02.043

Lekunberri, I., Balcazar, J.L., Borrego, C.M., 2018. Metagenomic exploration reveals a marked

change in the river resistome and mobilome after treated wastewater discharges. Environ. Poll. 234, 538-542. https://doi.org/10.1016/j.envpol.2017.12.001

López-Gutiérrez, J.C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J. Microbiol. Methods. 57, 399–407. https://doi.org/10.1016/j.mimet.2004.02.009

Lübbert, C., Baars, C., Dayakar, A., Lippman, N., Rodloff, A.C., Kinzig, M., Sörgel, F., 2017. Environmental pollution with antimicrobial agents from bulk drug manufacturing industries in Hyderabad, South India, is associated with dissemination of extended-spectrum beta-lactamase and carbapenemase-producing pathogens. Infection. 45, 479-491. https://doi.org/10.1007/s15010-017-1007-2

Marathe, N.P., Regina, V.R., Walujkar, S.A., Charan, S.S., Moore, E.R.B., Larsson, D.G.J., Shouche, Y.S., 2013. A treatment plant receiving waste water from multiple bulk drug manufacturers is a reservoir for highly multi-drug resistant integron-bearing bacteria. PLOS One. 8, 1-10. https://doi.org/10.1371/journal.pone.0077310

Marathe, N.P., Pal, C., Gaikwad, S.S., Jonsson, V., Kristiansson, E., Larsson, D.G.J., 2017. Untreated urban waste contaminates Indian river sediments with resistance genes to last resort antibiotics. Water Res. 124, 388-397. https://doi.org/10.1016/j.watres.2017.07.060

Marti, E., Huerta, B., Rodríguez-Mozaz, S., Barcelo, D., Jofre, J., Balcázar, J.L., 2014. Characterization of ciprofloxacin-resistant isolates from a wastewater treatment plant and its receiving river. Wat. Res. 61, 67-76. https://doi.org/10.1016/j.watres.2014.05.006

McLellan, S.L., Huse, S.M., Mueller-Spitz, S.R., Andreishcheva, E.N., Sogin, M.L., 2010. Diversity and population structure of sewage derived microorganisms in wastewater treatment plant influent. Environ. Microbiol. 12, 378-392. https://doi.org/10.1111/j.1462-2920.2009.02075.x

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957-995. https://doi.org/10.1016/j.watres.2012.11.027

Milaković, M., Vestergaard, G., González-Plaza, J.J., Petrić, I., Šimatović, A., Senta, I., Kublik, S., Schloter, M., Smalla, K., Udiković-Kolić, N., 2019. Pollution from azithromycin-manufacturing promotes macrolide-resistance gene propagation and induces spatial and seasonal bacterial community shifts in receiving river sediments. Environ. Int. 123, 501–511. https://doi.org/10.1016/j.envint.2018.12.050

Naas, T., Benaoudia, F., Massuard, S., Nordmann, P., 2000. Integron-located VEB-1 extended spectrum β-lactamase gene in a *Proteous mirabilis* clinical isolated from Vietnam. J. Antimicrob. Chemoter. 46, 703-711. https://doi.org/10.1093/jac/46.5.703

Narciso-da-Rocha, C., Vaz-Moreira, I., Manaia, C.M., 2014. Genotypic diversity and antibiotic resistance in *Sphingomonadaceae* isolated from hospital tap water. Sci. Total. Environ. 466-467, 127-135. https://doi.org/10.1016/j.scitotenv.2013.06.109

Newman, M.E.J., 2006. Modularity and community structure in networks. PNAS. 103, 8577–8582. https://doi.org/10.1073/pnas.0601602103

Nonaka, L., Maruyama, F., Miyamoto, M., Miyakoshi, M., Kurokawa, K., Masuda, M., 2012.

Novel conjugative transferable multiple drug resistance plasmid pAQU1 from *Photobactericum damselae* subsp. *damselae* isolated from marine aquaculture environment. Microbes Environ. 27, 263-272. https://doi.org/10.1264/jsme2.ME11338

Nnadzoie, C.F., Odume, O.N., 2019. Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. Environ. Pollut. 254, 1-15. https://doi.org/10.1016/j.envpol.2019.113067

O'Neill, J., 2016. The Review on Antimicrobial Resistance. Final Report.

Osinska, A., Korzeniewska, E., Harnisz, M., Niestepski, S., 2017. The prevalence and characteriaztion of antibiotic-resistant and virulent *Escherichia coli* strains in the municipal wastewater system and their environmental fate. Sci. Total Environ. 577, 367-375. https://doi.org/10.1016/j.scitotenv.2016.10.203

Piotrowska, M., Przygodzińska, D., Matyjewicz, K., Popowska, M., 2017. Occurrence and variety of β-lactamase genes among *Aeromonas* spp. isolated from urban wastewater treatment plant. Front. Microbiol. 8, 1-12. https://doi.org/10.3389/fmicb.2017.00863

Poirel, L., Rodriguez-Martinez, J.M., Mammeri, H., Liard, A., Nordmann, P., 2005. Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob. Agents Chemoter. 49, 3523-3525. https://doi.org/10.1128/AAC.49.8.3523-3525.2005

Rafraf, I.D., Lekunberri, I., Sànchez-Melsío, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353-358. https://doi.org/10.1016/j.envpol.2016.10.062

Rodríguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Melsió-Sànchez, A.,

Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234-242. https://doi.org/10.1016/j.watres.2014.11.021

Sabri, N.A., Schmitt, H., Van der Zaan, B., Gerritsen, H.W., Zuidema, T., Rijnaarts, H.H.M., Lagenhoff, A.A.M., 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2018.03.004

Sáenz, J.S., Marques, T.V., Barone, R.S.C., Cyrino, J.E.P., Kublik, S., Nesme, J., Rath, S., Vestergaard, G., 2019. Oral administration of antibiotics increased the potential mobility of bacterial resistance genes in the gut of the fish *Piaractus mesopotamicus*. Microbiome. 7, 1-14. https://doi.org/10.1186/s40168-019-0632-7

Seiler, C., Berendonk, T.U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3, 1-10. https://doi.org/10.3389/fmicb.2012.00399

Senta, I., Terzic, S., Ahel, M., 2013. Occurrence and fate of dissolved and particulate antimicrobials in municipal wastewater treatment. Water Res. 47, 705–714. https://doi.org/10.1016/j.watres.2012.10.041

Senta, I., Terzić, S., Ahel, M., 2008. Simultaneous determination of sulfonamides, fluoroquinolones, macrolides and trimethoprim in wastewater and river water by LC-tandem-MS. Chromatographia. 68, 747–758. https://doi.org/10.1365/s10337-008-0780-6

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski,

B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498-2504. https://doi.org/10.1101/gr.1239303

Sidrach-Cardona, R., Hijosa-Valsero, M., Marti, E., Balcázar, J.L., Becares, E., 2014. Prevalence of antibiotic-resistant fecal bacteria in a river impacted by both an antibiotic production plant and urban treated discharges. Sci. Total Environ. 488-489, 220-227. https://doi.org/10.1016/j.scitotenv.2014.04.100

Su, J.Q., Wei, B., Ou-Yang, W.Y., Huang, F.Y., Zhao, Y., Xu, H.J., Zhu, Y.G., 2015. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ. Sci. Technol. 49, 7356-7563. https://doi.org/10.1021/acs.est.5b01012

Šimatović, A., Udiković-Kolić, N., 2019. Antibiotic Resistance in Pharmaceutical Industry Effluents and Effluent-Impacted Environments, in: The Handbook of Environmental Chemistry. Springer Berlin, Heidelberg, pp. 1-22. https://doi.org/10.1007/698_2019_389

Vaz-Moreira, I., Nunes, O.C., Manaia, C.M., 2011. Diversity and antibiotic resistance of *Sphingomonadaceae* isolates from drinking water. Appl. Environ. Microbiol. 77, 5697-5706. https://doi.org/10.1128/AEM.00579-11

Wenthworth, C.K., 1922. A scale of grade and class term for sifting sediments. J. Geol. 30, 377–392. https://doi.org/10.2307/30063207

Wibberg, D., Salto, I.P., Eikmeyer, F.G., Maus, I., Winkler, A., Nordmann, P., Pühler, A., Poirel, L., Schlüter, A., 2018. Complete genome sequencing of *Acinetobacter baumanii* strain

K50 discloses the large conjugative plasmid pK50a encoding carbapenemase OXA-23 and extended-spectrum β-lactamase GES-11. Antimicrob. Agents Chemoter. 62, 1-12. https://doi.org/10.1128/AAC.00212-18

Wong, D., Nielsen, T.B., Bonomo, R.A., Pantapalangkoor, P., Luna, B., Spellberg, B., 2017. Clinical and pathophysiological overview of *Acinetobacter* infections: a century of challenges. Clin. Microbiol. Rev. 30, 409-447. https://doi.org/10.1128/CMR.00058-16

Yang, F., Mao, D., Zhou, H., Luo, Y., 2016. Prevalence and fate of carbapenemase genes in wastewater treatment plant in Northern China. PLOS One. 11, 1-14. https://doi.org/10.1371/journal.pone.0156383

Yang, Y., Zhang, T., Zhang, X.X., Liang, D.W., Zhang, M., Gao, D.W., Zhu, H.G., Huang, Q.G., Fang, H.H.P., 2012. Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America. Appl. Microbiol. Biotechnol. 95, 1351-1358. https://doi.org/10.1007/s00253-011-3810-5

Zhai, W., Yang, F., Mao, D., Luo, Y., 2016. Fate and removal of various antibiotic resistance genes in typical pharmaceutical wastewater treatment systems. Environ. Sci. Pollut. Res. 23, 12030–12038. https://doi.org/10.1007/s11356-016-6350-9

Zhou, Y.Q., Meng, J., Zhang, M., Chen, S.Q., He, B., Zhao, Z., Li, Q.F., Zhang, S., Wang, T.Y., 2019. Which type of pollutants need to be controlled with priority in wastewater treatment plants: Traditional or emerging pollutants? Environ. Int. 131, 1-13. https://doi.org/10.1016/j.envint.2019.104982

Figure Captions:

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

Figure 2. NMDS analysis based on Bray-Curtis dissimilarity 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 were 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.

Figure 3. Heat maps of the 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. UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge; WW, effluent.

Figure 4. Network analysis revealing co-occurrence patterns among analyzed ARGs and their potential bacterial hosts. The nodes were coloured according to the phylum affiliation. A connection represents strong (Spearman's correlation coefficient (ρ) > 0.7) and significant ($p < 0.01$) correlation. Node size was weighted according to the number of connections (i.e. degree) and edges were weighted according to the correlation coefficient.

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 potentiaI competing interests:

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 (mg/kg dry sediment)		
				UP	DW0	DW3000
Sulfonamides	Sulfadiazine	SDZ	Winter	0.007	0.26	0.69
			Summer	0.02	0.04	0.16
	Sulfamethazine	SMZ	Winter	0.03	0.19	0.48
			Summer	0.03	0.09	0.40
Diaminopyridines	Trimethoprim	TMP	Winter	0.04	0.37	0.28
			Summer	0.04	5.08	0.30
Macrolides	Azithromycin	AZI	Winter	0.01	0.15	0.07
			Summer	0.01	0.39	0.35
Total antibiotics, ∑			Winter	0.09	0.97	1.52
			Summer	0.10	5.60	1.21

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

Graphical abstract

Highlights:

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

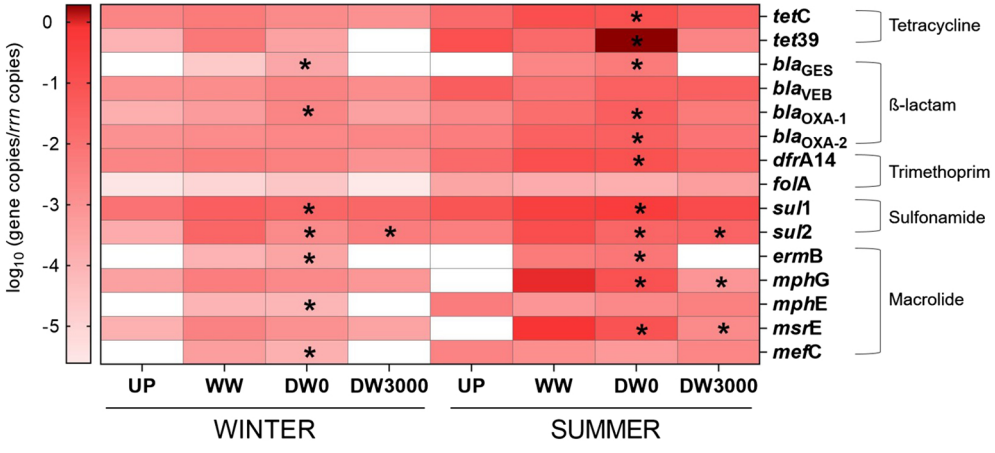
log10 (gene copies/rrn copies)
0
-1
-2
-3
-4
-5
tetC
tet39
blaGES
blaVEB
blaOXA-1
blaOXA-2
dfrA14
folA
sul1
sul2
ermB
mphG
mphE
msrE
mefC
Tetracycline
ß-lactam
Trimethoprim
Sulfonamide
Macrolide
UP
WW
DW0
DW3000
UP
WW
DW0
DW3000
WINTER
SUMMER

Figure 1

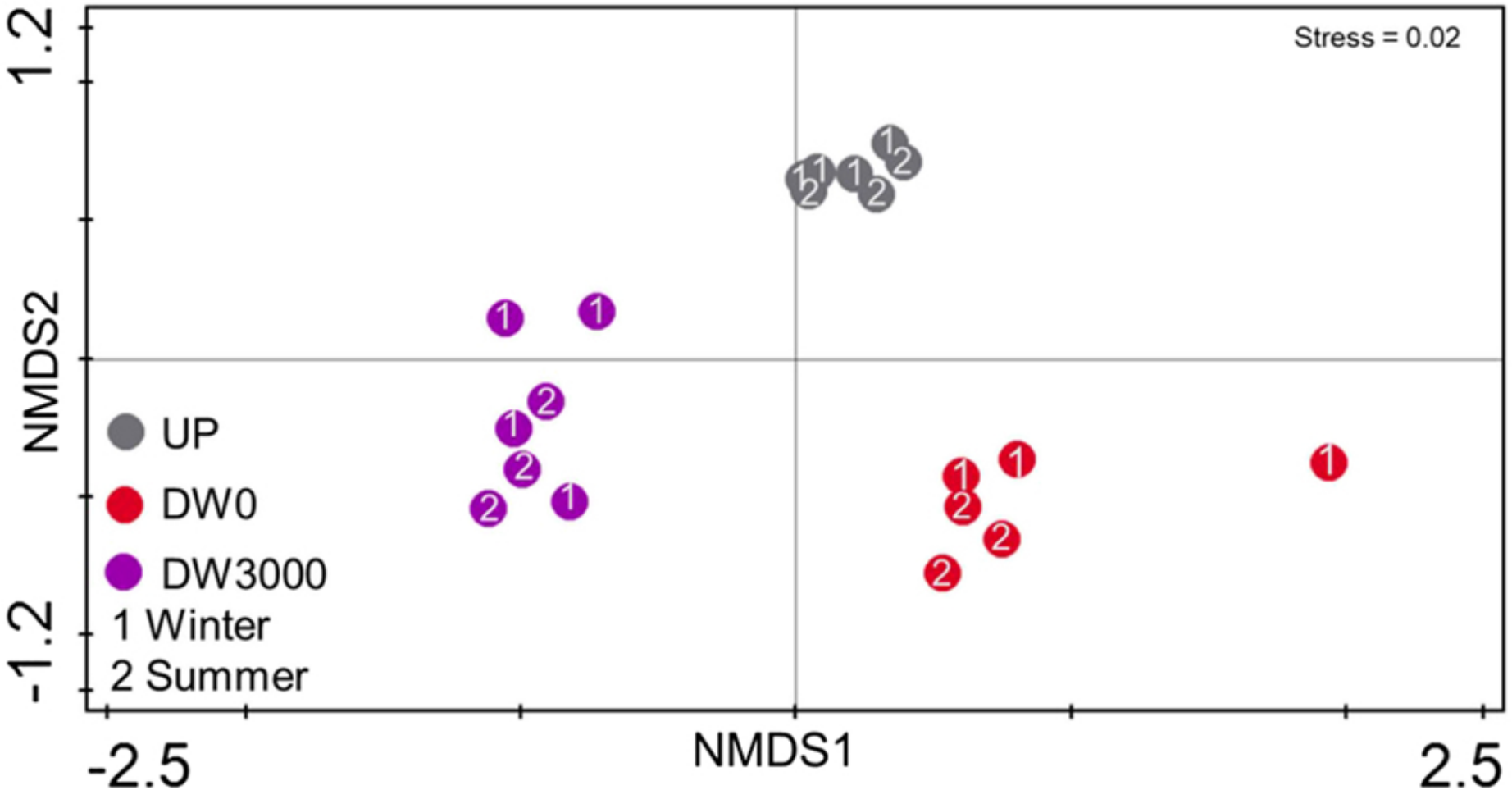
Stress = 0.02
1.2
-1.2
NMDS2
-2.5
NMDS1
2.5
UP
DW0
DW3000
1 Winter
2 Summer

Figure 2

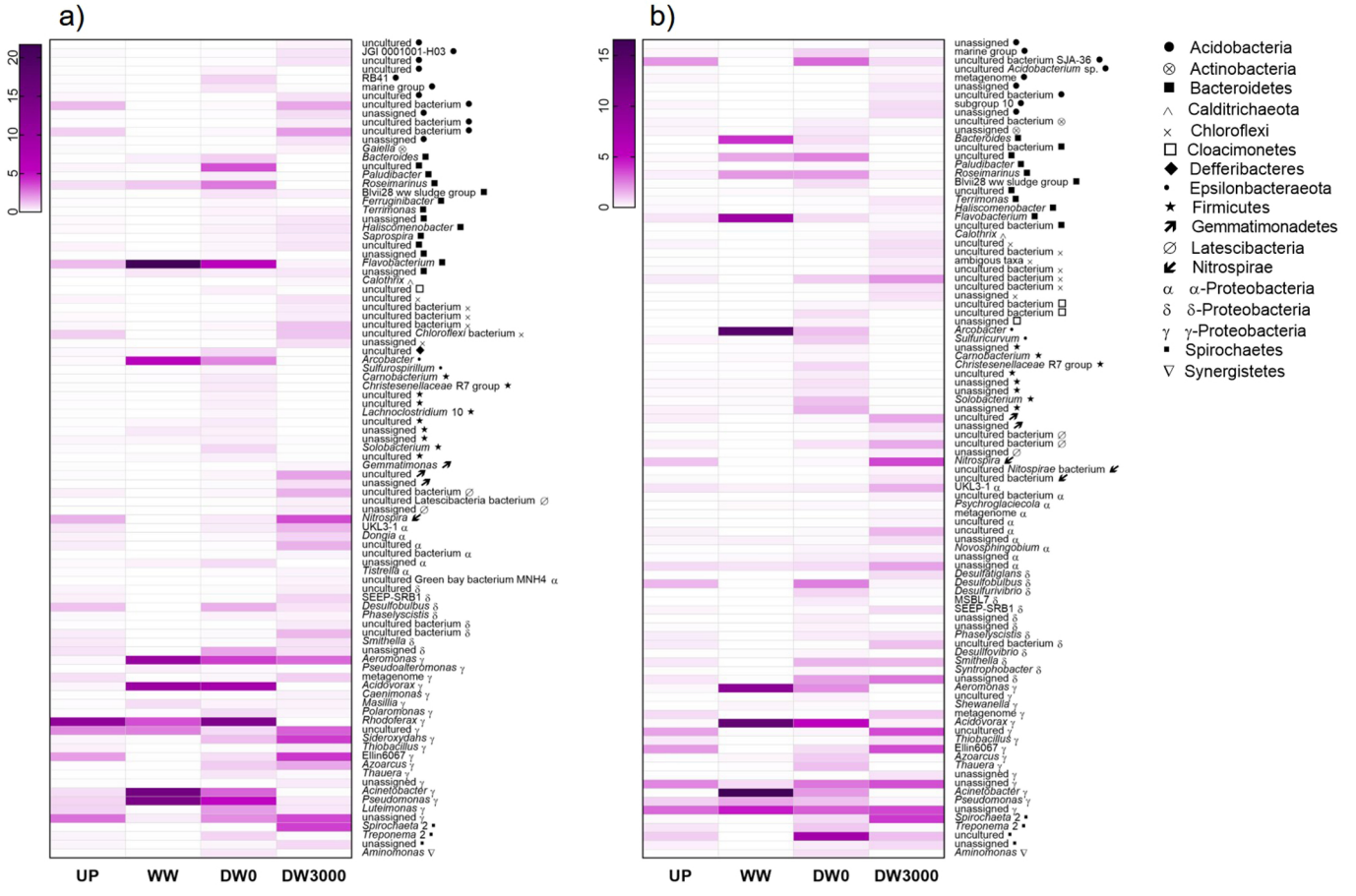
a)
b)
0
5
10
15
20
UP
WW
DW0
DW3000
uncultured
JGI 0001001-H03
marine group
RB41
uncultured bacterium
unassigned
Gaiella
Bacteroides
Paludibacter
Roseimarinus
Blvi28 ww sludge group
Ferruginibacter
Terrimonas
Haliscomenobacter
Saprospira
Flavobacterium
Calothrix
Chloroflexi bacterium
Arcobacter
Sulfurospirillum
Carnobacterium
Christesenellaceae R7 group
Lachnoclostridium 10
Solobacterium
Gemmatimonas
Latescibacteria bacterium
Nitrospira
UKL3-1
Dongia
Tistrella
Green bay bacterium MNH4
SEEP-SRB1
Desulfobulbus
Phaselyscistis
Smithella
Aeromonas
Pseudoalteromonas
metagenome
Acidovorax
Caenimonas
Masillia
Polaromonas
Rhodoferax
Sideroxydans
Thiobacillus
Ellin6067
Azoarcus
Thauera
Acinetobacter
Pseudomonas
Luteimonas
Spirochaeta 2
Treponema 2
Aminomonas
SJA-36
Acidobacterium sp.
subgroup 10
Terrimonas
Haliscomenobacter
Flavobacterium
Calothrix
Arcobacter
Sulfurcurvum
Solobacterium
Nitrospira
Nitrospirae bacterium
Psychroglaciecola
Novosphingobium
Desulfatiglans
Desulfobulbus
Desulfurivibrio
MSBL7
SEEP-SRB1
Phaselyscistis
Desulfovibrio
Smithella
Syntrophobacter
Aeromonas
Shewanella
Acidovorax
Thiobacillus
Ellin6067
Azoarcus
Thauera
Acinetobacter
Pseudomonas
Spirochaeta 2
Treponema 2
Aminomonas
Acidobacteria
Actinobacteria
Bacteroidetes
Calditrichaeota
Chloroflexi
Cloacimonetes
Defferibacteres
Epsilonbacteraeota
Firmicutes
Gemmatimonadetes
Latescibacteria
Nitrospirae
α-Proteobacteria
δ-Proteobacteria
γ-Proteobacteria
Spirochaetes
Synergistetes

Figure 3

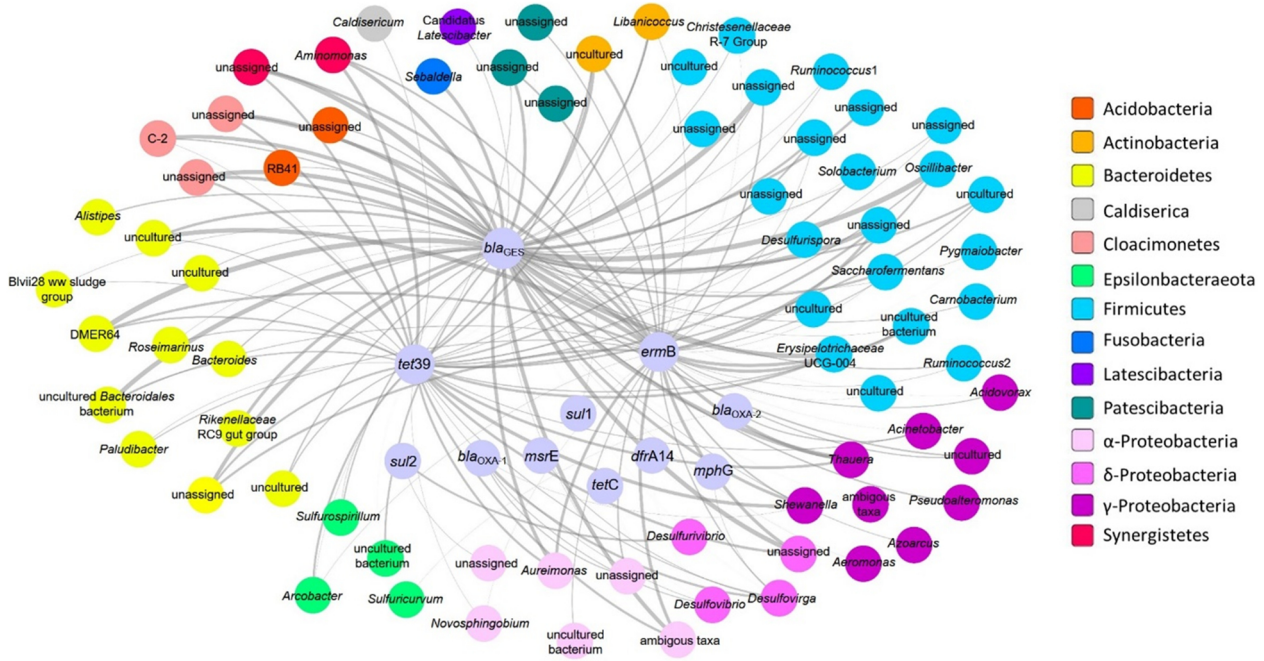
Caldisericum
Candidatus Latescibacter
unassigned
Libanicoccus
Christensenellaceae R-7 Group
Aminomonas
Sebaldella
uncultured
unassigned
unassigned
unassigned
unassigned
Ruminococcus1
unassigned
unassigned
C-2
RB41
unassigned
unassigned
unassigned
unassigned
unassigned
Solobacterium
Oscillibacter
unassigned
uncultured
Alistipes
uncultured
blaGES
Desulfurispora
unassigned
Pygmaiobacter
Saccharofermentans
Blvii28 ww sludge group
uncultured
uncultured
Carnobacterium
uncultured
bacterium
DMER64
Roseimarinus
Bacteroides
tet39
ermB
Erysipelotrichaceae UCG-004
Ruminococcus2
uncultured
Acidovorax
uncultured Bacteroidales bacterium
sul1
blaOXA-2
Rikenellaceae RC9 gut group
Acinetobacter
Paludibacter
sul2
blaOXA-1
msrE
dfrA14
mphG
Thauera
uncultured
unassigned
uncultured
tetC
Shewanella
ambigous taxa
Pseudoalteromonas
Sulfurospirillum
Desulfurivibrio
Aeromonas
Aeromonas
uncultured bacterium
unassigned
Aureimonas
unassigned
Arcobacter
Sulfuricurvum
Desulfovibrio
Desulfovirga
Novosphingobium
uncultured bacterium
ambigous taxa
Acidobacteria
Actinobacteria
Bacteroidetes
Caldiserica
Cloacimonetes
Epsilonbacteraeota
Firmicutes
Fusobacteria
Latescibacteria
Patescibacteria
α-Proteobacteria
δ-Proteobacteria
γ-Proteobacteria
Synergistetes

Figure 4