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Abstract

Antibiotic resistance is an emerging global health crisis, driven largely by overuse and misuse of antibiotics. However, there are examples in which the production of these antimicrobial agents has polluted the environment with active antibiotic residues, selecting for antibiotic resistant bacteria and the genes they carry. In this work, we have used shotgun metagenomics to investigate the taxonomic structure and resistance gene composition of sludge communities in a treatment plant in Croatia receiving wastewater from production of the macrolide antibiotic azithromycin. We found that the total abundance of antibiotic resistance genes was three times higher in sludge from the treatment plant receiving wastewater from pharmaceutical production than in municipal sludge from a sewage treatment plant in Zagreb. Surprisingly, macrolide resistance genes did not have higher abundances in the industrial sludge, but genes associated with mobile genetic elements such as integrons had. We conclude that at high concentrations of antibiotics, selection may favor taxonomic shifts towards intrinsically resistant species or strains harboring chromosomal resistance mutations rather than acquisition of mobile resistance determinants. Our results underscore the need for regulatory action also within Europe to avoid release of antibiotics into the environment.

Keywords

Antibiotic resistance, Community structure, Macrolides, Pharmaceutical production, Wastewater treatment

1. Introduction

Rising levels of antibiotic resistance are gradually impairing our ability to treat infectious diseases, perform surgery and utilize immuno-suppressive therapies, shaking the foundations of modern healthcare (French, 2010; Review on Antimicrobial Resistance, 2016). While extensive use and overuse of antibiotics in the clinics are likely the ultimate drivers of resistance accumulation in human pathogens, it has in the last decade been recognized that the external environment is likely to play an important role in both transmission of resistant bacteria and development of novel resistance phenotypes (Finley *et al.*, 2013; Berendonk *et al.*, 2015; Bengtsson-Palme *et al.*, 2018b; Larsson *et al.*, 2018). Selective pressure from antibiotics plays a critical role in both these processes (Bengtsson-Palme *et al.*, 2018b). Discharges from pharmaceutical manufacturing facilities have repeatedly been shown to provide conditions where antibiotics reach concentrations that are selective for resistance enrichment (Larsson, 2014). Increased numbers of resistant bacteria and resistance genes have indeed been found in environments impacted by antibiotic production waste, for example in China (Li *et al.*, 2010), Korea (Sim *et al.*, 2011) and India (Kristiansson *et al.*, 2011; Bengtsson-Palme *et al.*, 2014; Marathe *et al.*, 2013). However, the problem of active antibiotic substances being released from pharmaceutical production is not confined to Asia. Bielen *et al.* (2017) recently showed high, mg/L concentrations of macrolide antibiotics (azithromycin and erythromycin) in wastewaters from a Croatian pharmaceutical manufacturing facility synthesizing the macrolide antibiotic azithromycin. In addition, high levels of azithromycin-resistant bacteria and known (*msr, mph, mef*) as well as novel (*erm*) macrolide-resistance genes were found in these wastewaters and the receiving river sediments using functional metagenomics (González-Plaza *et al.*, 2017).

Macrolides constitute a diverse class of natural and semisynthetic antibiotic compounds, which are widely used in both human and veterinary medicine (European Medicines Agency, 2018). Together with cephalosporins, macrolides had the second-highest usage according to the World Health Organization (WHO) report on surveillance of antibiotic consumption in European region in 2016-2018 (World Health Organization, 2018). Furthermore, in order to optimize antibiotic use and reduce antibiotic resistance, the WHO has recently named certain antibiotic classes, including macrolides, as highest priority critically important antibiotics for human medicine (World Health Organization, 2017). The most commonly used macrolides in human medicine are erythromycin, azithromycin and clarithromycin (Keskar and Jugade, 2015). They are effective against Gram-positive as well as against some Gram-negative bacteria and are often used to treat community-acquired respiratory tract infections, skin and soft tissue infections, sexually transmitted diseases, shigellosis and salmonellosis (Fyfe *et al.*, 2016; Keskar and Jugade, 2015). Macrolides inhibit protein synthesis by binding to the 50S ribosomal subunit, and resistance to this class of antibiotics is mainly attributed to target site modification (*erm* genes), active efflux (*mef*, *msr* genes) or modification of the drug itself (*ere*, *mph* genes) (Fyfe *et al.*, 2016).

Wastewater treatment plants (WWTPs) have been proposed as hot spots for dissemination of antibiotics and antibiotic resistance determinants into the aquatic environment (Michael *et al.*, 2013; Rizzo *et al.*, 2013; Guo *et al.*, 2017). Activated sludge treatment is a widely used technology in WWTPs for treating both municipal and industrial wastewaters. In the case of wastewaters from antibiotic production, which often contain high levels of antibiotics (Larsson *et al.*, 2007; Bielen *et al.*, 2017), such biological treatment can result in massive enrichment of antibiotic resistant bacteria, resistance genes and associated mobile elements and, consequently, alteration of the sludge

103 microbial community due to selection by the antibiotic residues (Marathe *et al.*, 2013; Wang *et al.*,
104 2015). Therefore, industrial WWTPs are "worst case" scenarios for selection of antibiotic resistance
105 in the environment and should be studied more closely. In this work, we used shotgun
106 metagenomics to compare sludge samples from a WWTP receiving wastewaters from a Croatian
107 azithromycin manufacturing facility (Bielen *et al.*, 2017) and sludge from a WWTP located in Zagreb
108 which receives mainly municipal wastewater, to better understand how antibiotic exposure impacts
109 the diversity and abundance of known resistance genes, mobile genetic elements and microbial
110 organisms. We found that sludge from the industrial WWTP harbored around three times higher
111 abundances of resistance genes than the municipal sewage sludge, with particularly large
112 enrichments of aminoglycoside, amphenicol and sulfonamide resistance genes. Surprisingly, the
113 overall abundance of macrolide resistance genes was not higher in the industrial sludge. These
114 findings highlight that antibiotic production in European settings also contributes to the
115 development of antibiotic resistance and indicate potential for co-selection of resistance genes to
116 a variety of antibiotic classes.

117 ## 2. Materials and Methods

118 *2.1 Sampling and DNA extraction*

119 Activated sludge samples were collected from the aeration tanks of two WWTPs: one receiving
120 wastewater from a pharmaceutical manufacturing facility and another receiving wastewater from
121 the city of Zagreb. The industrial WWTP receives a combination of technological (manufacturing
122 of active pharmaceutical ingredients, mainly azithromycin) and sanitary wastewaters from the
123 Croatian pharmaceutical company Pliva and utilizes a membrane bioreactor system for their

124 treatment. This system is designed to treat industrial wastewaters previously pre-treated with
125 equalization and neutralization, and consists of the aerated and anoxic tanks for the removal of
126 organic matter and nitrification/denitrification, a membrane zone for liquid/solid separation and
127 sludge digestive basins. The Zagreb WWTP receives mainly municipal sewage plus a small
128 contribution from hospitals and industries (not from macrolide synthesis; about 1,000,000
129 population equivalents). It includes full mechanical and biological treatment based on conventional
130 activated sludge treatment. Approximately one-liter grab samples of the mixed liquor (i.e a mixture
131 of wastewater and activated sludge within the aeration tank) were collected from the Zagreb
132 WWTP in November 2017 while samples from the industrial WWTP were collected in January
133 2016. Three samples from different locations in the aeration tank were collected from each
134 treatment plant. All samples were collected in sterile plastic containers and with appropriate
135 permissions from WWTP authorities. The samples were stored on ice during transport to the
136 laboratory.

137 Total genomic DNA was extracted from concentrated sludge samples (0.25 g of the pellet after
138 centrifugation of the mixed liquor at 4000 x *g* for 10 min at room temperature) using the Power
139 Soil DNA isolation kit (MOBio, USA) according to the manufacturer´s recommendations. The
140 extraction yield and quality of the DNA were verified by spectrophotometry (Nanodrop BioSpec
141 Nano, Shimadzum, Japan) and the quantity was verified by fluorimetry (Qubit Fluorometer 3.0,
142 Thermo Fisher Scientific, USA). All extractions were stored at -20°C until used.

143 *2.2 Chemical analysis*

144 Chemical analyses of different antibiotic classes were performed in both solid and aqueous phases
145 of sampled mixed liquor from the aeration tanks. The samples were defrosted and centrifuged to

146 separate solid and aqueous phases. Internal standards (isotope labelled antibiotics: clarithromycin,
147 sulfamethoxazole, trimethoprim and clindamycin) were added to samples prior to the analysis. Due
148 to expected high concentrations of macrolides, we used a modified analytical method based on
149 previously published work (Grabic *et al.*, 2012; Golovko *et al.*, 2016). Briefly, 10 µl of aqueous
150 samples were directly injected onto the analytical column (HypersilGold aQ, 2.1 mm ID x 50 mm
151 length, 5 µm particles, ThermoScientific, USA). We used three different levels of dilution: no
152 dilution, 10 times and 100 times diluted samples. Solids were extracted using ultrasonic extraction
153 with mixtures of water/acetonitrile and water/acetonitrile/isopropanol in two steps (Golovko *et*
154 *al.*, 2016). Extracts were combined and later analyzed using 10 µl injection onto the same column
155 as aqueous samples. Analogically to water samples we had to use multiple extract dilution for
156 compounds at extremely high concentrations (azithromycin). Due to the complexity of the matrix,
157 we assured selectivity of mass spectrometric detection using electrospray ionization hybrid
158 quadrupole/orbital trap mass spectrometer QExactive HF (ThermoScientific, USA) operated in
159 both full scan and high-resolution product scan (HRPS) instead of conventional QqQ. Detailed
160 descriptions of the MS method have been reported in Grabicova *et al.* (2018).

161 *2.3 Sequencing*

162 DNA sequencing of the six samples was performed at Science for Life Laboratories (Stockholm,
163 Sweden). Clustering was done by cBot and samples were sequenced in one lane of an Illumina
164 HiSeq2500 instrument (HiSeq Control Software 2.2.58/RTA 1.18.64) with a 2x126 setup using
165 HiSeq SBS Kit v4 chemistry. The BCL to FASTQ conversion was performed using the CASAVA
166 software suite. The sequence data have been deposited in the European Nucleotide Archive under
167 the accession PRJEB26809.

2.4 Bioinformatic analysis

FASTQ files were trimmed for low quality bases and adapters using TrimGalore! with the settings "--retain_unpaired --paired --phred33 -e 0.1 -q 28 -O 10", removing reads shorter than 20 bp (the default setting) after quality trimming (Babraham Bioinformatics, 2012). Conversions between FASTQ and FASTA formats were done using Pefcon, part of the PETKit (http://microbiology.se/software/petkit). The samples were analyzed for taxonomic composition using Metaxa2 (version 2.2 beta 9) with default settings (Bengtsson-Palme *et al.*, 2015b) and further processed using Metaxa2 Diversity Tools (Bengtsson-Palme *et al.*, 2016b). Antibiotic resistance genes were quantified by mapping quality-filtered reads to the ResFinder database (Zankari *et al.*, 2012) using Usearch (version 8.0.1445) with the "--usearch_global" option and identity cutoff 0.9 (Edgar, 2010). As the resistance genes identified by González-Plaza *et al.* (2017) were not present in the ResFinder database, their sequences were downloaded from GenBank, translated to amino acid sequences using Prodigal (Hyatt *et al.*, 2010) and the abundances of those resistance genes in the samples were quantified using Usearch as above. Integrase and transposon sequences were identified by mapping to a custom database (Supplementary Item 1), using Usearch with the above options. To identify known plasmid sequences in the data, the reads were mapped to the NCBI Plasmid database (downloaded on 2019-05-14) using Bowtie2 and the options "-f -p 16 --no-unal --no-hd --no-sq". The mapped read information was added to a FARAO database (Hammarén *et al.*, 2016) for quantification and visualization. A plasmid was considered detected if at least ten reads mapped to it from a sequencing library. A custom database of 23S rRNA sequences with known resistance mutations was generated from sequences in the CARD database (Jia *et al.*, 2016) together with the corresponding wildtype sequences using Mumame (version 1.0) (Magesh *et al.*, 2018). For this database, only cutouts around the resistance mutation 55 nucleotides upstream and

191 downstream were included. Reads were mapped to the database using Mumame in the Usearch
192 mode and the following options "-n -c 0.98 --alnout". Comparisons were made between the
193 matches to mutated sequences and wildtype sequences using the R script provided with the
194 Mumame software.

195 *2.5 Statistical analysis*

196 The data was analyzed in R version 3.3.2 using the Vegan package (version 2.4-1) (R Core Team,
197 2016; Oksanen *et al.*, 2016). Unless otherwise specified, statistical differences were assessed using
198 overdispersed Poisson generalized linear models, as this has been suggested in previous literature
199 to provide good power and error control with only three replicates (Jonsson *et al.*, 2016; Bengtsson-
200 Palme *et al.*, 2017). Rarefied richness was used to describe the diversity of resistance genes, mobile
201 genetic elements and plasmids (Bengtsson-Palme, 2018). The metaxa2_uc utility, which tests
202 whether there is a significant difference between within-group and between-group Bray-Curtis
203 dissimilarities (Bengtsson-Palme *et al.*, 2016b), was used to assess differences in taxonomic
204 composition (default options).

205 ## 3. Results

206 *3.1 Chemical analysis*

207 Chemical analysis of the mixed liquor samples showed that the concentration of azithromycin
208 reached 1200 μg/L in the aqueous phase in the aeration tank of the industrial WWTP – 55 times
209 higher than concentrations generally found in the municipal WWTP – and 4300 ng/g in the sludge
210 (Table 1; Supplementary Table 1). Concentrations of erythromycin were lower (4.3 μg/L in
211 aqueous phase; not detected in sludge). The azithromycin concentrations in the industrial treatment

plant were well above measured inhibitory concentrations as well as concentrations predicted to drive antibiotic resistance development (Bengtsson-Palme and Larsson, 2016a).

Table 1. Average concentrations of macrolide antibiotics in mixed liquor collected from the aeration tanks of industrial and municipal wastewater treatment plants (WWTPs).

Compound	PNEC* (µg/L)	Activated sludge (ng/g)		Aqueous phase (µg/L)	
		Zagreb municipal WWTP	Industrial WWTP	Zagreb municipal WWTP	Industrial WWTP
Azithromycin	0.25	450	4300	22	1200
Erythromycin	1	<16	<37	<0.13	4.3

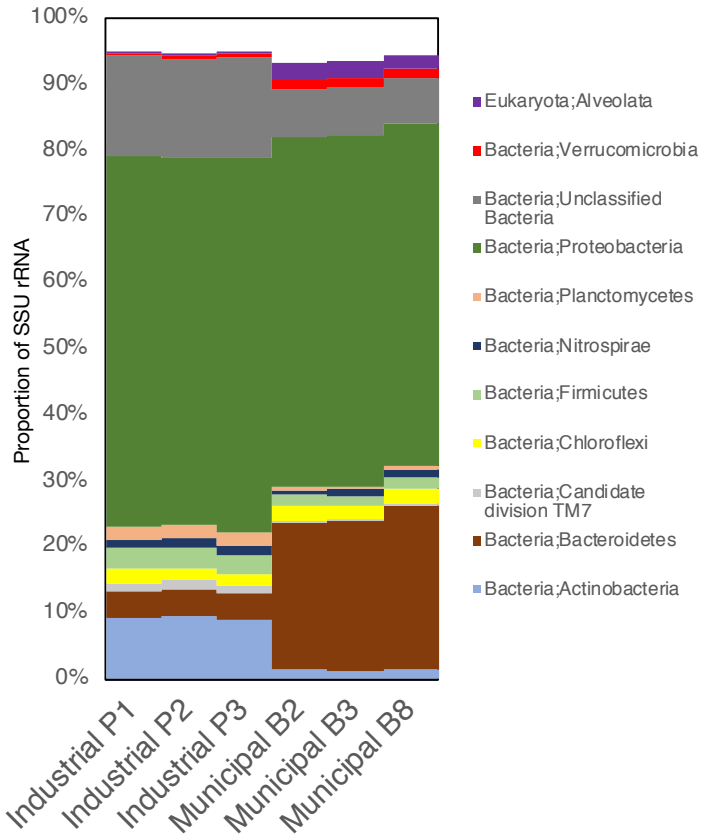
*PNEC, Predicted No-Effect Concentration (for resistance selection)

3.2 Effects on taxonomic composition

In total, we obtained 171 million paired reads from Illumina sequencing, corresponding to 24.9 to 33.1 million reads per sample. After quality filtering, a total of 170.4 million reads remained in the libraries, suggesting a very high-quality sequencing run. We detected between 11,306 and 14,408 SSU rRNA sequences in the samples. The number of SSU sequences per million reads were higher in the municipal samples (475.8 vs. 418.9, $p = 3.61 \times 10^{-6}$). This shift seems to be due to lower relative abundances of eukaryotes (which often carry large numbers of copies of the SSU genes) in the industrial samples (13-fold reduction; $p = 0.0062$). On the phylum level, the municipal sludge composition was in line with previously analyzed activated sludge samples (Bengtsson-Palme *et al.*, 2016a; Ju and Zhang, 2015). The relative abundance of Bacteriodetes was lower in the industrial sludge compared to the municipal, while Actinobacteria, Planctomyces and unclassifiable bacteria had higher abundances (Figure 1). The taxonomic composition at the genus level was very dissimilar between the two sample types ($p < 0.0001$; metaxa2_uc). In addition, the genus diversity

231 was significantly higher in municipal compared to industrial sludge (Student's t-test, p = 0.0007).
232 Interestingly, the difference in terms of Simpson's index was fairly small (0.956 for industrial, 0.968
233 for municipal).

Figure 1.



234

235 **Figure 1.** Taxonomic composition of municipal and industrial sludge samples at the Phylum level.

236 *Hyphomicrobium*, which was the fourth most abundant genus in industrial samples but had very low
237 abundance in municipal sludge, was one of the genera with most significantly higher abundance in
238 industrial sludge, together with e.g. *Xanthomonas* and *Dokdonella* (Supplementary Table 2).
239 *Acinetobacter*, *Roseiflexus*, *Sorangium* and *Flavobacterium* were among the genera significantly less
240 common in the industrial sludge. *Flavobacterium* and *Hyphomicrobium* were also the two genera most

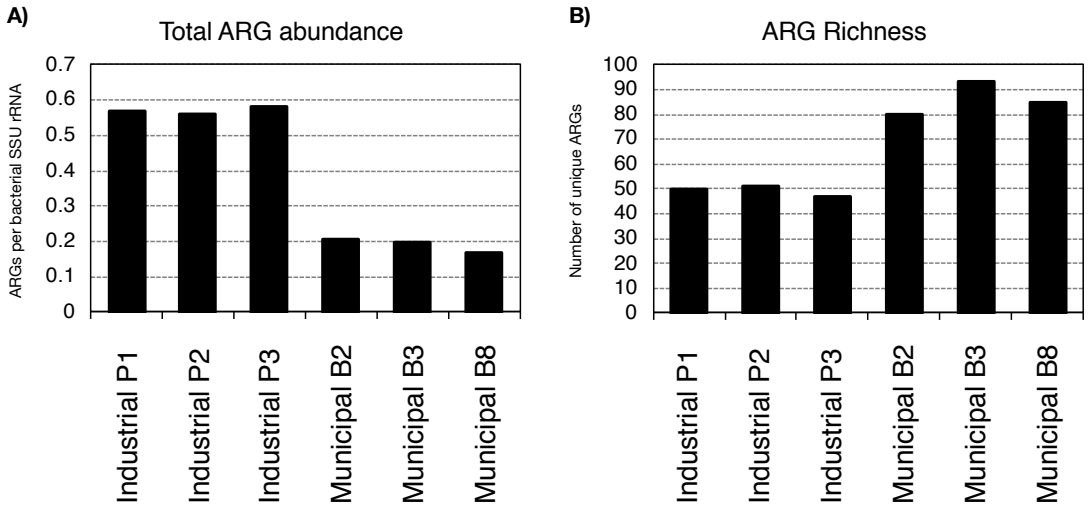
241 strongly driving the separation between the compositions of the sample types (Supplementary
242 Figure 1). Notably, many of the taxonomic groups with significantly different abundances could
243 not be classified to the genus level.

244 *3.3 Effects on antibiotic resistance gene abundances*

245 The total abundance of antibiotic resistance genes per 16S rRNA gene copy in sludge was about
246 three-fold higher in industrial compared to municipal samples ($p = 3.24x10^{-5}$; Figure 2A).
247 Interestingly, however, the total number of unique antibiotic resistance genes (i.e. resistance gene
248 richness) was lower in industrial compared to municipal samples ($p = 0.00136$; Figure 2B). This is
249 reflected in that only a small number of resistance genes accounted for the difference in total
250 abundance (Table 2), most prominently *sul1*, *flo*R, *sul2* and *aph(6)-Id*. This relatively small set of
251 enriched resistance genes also seem to be the main drivers of the differences observed at the
252 antibiotic class level (Figure 3A). After correction for multiple testing, we found that
253 aminoglycoside, amphenicol, sulfonamide, tetracycline and trimethoprim resistance genes were
254 significantly more common in the industrial sludge, while the macrolide-lincosamide-streptogramin
255 (MLS) class of genes showed significantly lower abundance in industrial compared to municipal
256 sludge. The latter observation was highly surprising as both the chemically measured compounds
257 characteristic for the production plant – azithromycin as a final product and erythromycin as a
258 precursor in synthesis – are macrolide antibiotics, and we would have expected the resistance
259 factors to macrolides to be more abundant in these settings. We therefore further investigated the
260 MLS resistance genes specifically to determine if there was a pattern that could explain their overall
261 lower abundances despite a strong selective pressure for macrolide resistance. We then found that
262 there was a contrasting pattern in the two most abundant MLS resistance genes, where *erm(F)* had

263 higher abundance in the industrial samples, while *mph(E)* was more abundant in the municipal
264 samples (Figure 3B). All other significant differences corresponded, surprisingly, to lower
265 abundances in the industrial samples, but they occurred in comparatively low-abundant resistance
266 genes.

Figure 2.



267

268 **Figure 2.** Total abundance (A) and richness (B) of antibiotic resistance genes (ARGs) in the industrial and municipal
269 sludge samples.

270 To investigate if this was due to MLS resistance genes not present in the ResFinder database, we
271 also mapped the data to the MLS resistance genes identified by González-Plaza *et al.* (2017) from
272 wastewaters of the same treatment plant and the receiving river sediments. This analysis confirmed
273 the same pattern (Figure 3C), suggesting that the lower abundances were not due to increased
274 prevalence of uncharacterized resistance genes. We attempted to investigate if there was instead a
275 higher abundance of chromosomal macrolide and erythromycin resistance mutations by mapping
276 all reads to 23S rRNA sequences containing known resistance mutations. While we could detect

277 twelve different genes containing mutations in either of the industrial or municipal samples, so few
278 reads mapped with high identity that the results were inconclusive in terms of resistance selection
279 (Supplementary Table 3).

280

281 **Table 2.** Antibiotic resistance genes (ARGs) with significantly different relative abundance per 16S rRNA in the
282 industrial and municipal sludges

ARGs	Industrial abundance	Municipal abundance	Adjusted p-value	Rank Industrial	Rank Municipal	Abundance difference
sul1	1.97E-04	2.22E-05	0.00038	1	2	8.9x
*flo*R	1.72E-04	3.58E-06	0.00049	2	12	48.1x
aph(6)-Id	5.32E-05	3.27E-06	0.0013	4	14	16.3x
blaOXA-2	3.08E-05	2.01E-06	0.0013	8	18	15.3x
strA	5.04E-05	5.90E-06	0.0013	5	7	8.5x
sul2	6.35E-05	8.95E-06	0.0013	3	4	7.1x
ant(3'')-Ia	2.03E-05	8.11E-06	0.0023	9	5	2.5x
aadA2	1.85E-05	2.53E-06	0.0034	10	16	7.3x
tet(G)	3.53E-05	1.84E-06	0.0034	7	22	19.2x
erm(F)	4.56E-05	1.15E-05	0.0040	6	3	4.0x
mph(E)	1.62E-05	1.13E-04	0.011	11	1	-7.0x
tet(31)	1.48E-05	6.09E-08	0.041	12	122	242.8x

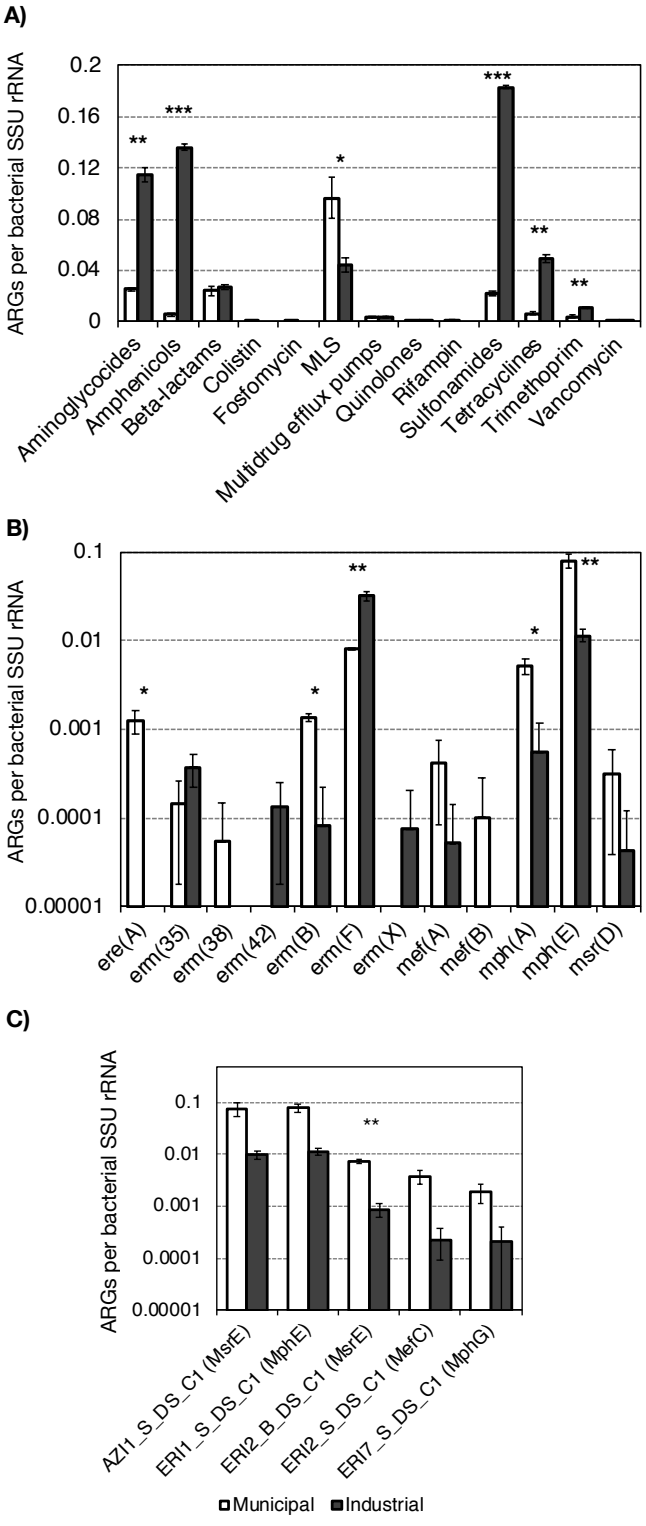
283

284

285

Figure 3. (A) Total abundance of antibiotic resistance genes (ARGs) per 16S rRNA gene copy in municipal and industrial samples divided by antibiotic classes. MLS corresponds to Macrolide-Lincosamide-Streptogramin antibiotics. (B) Abundances of macrolide resistance genes per 16S rRNA gene copy. (C) Abundances of macrolide resistance genes identified by functional metagenomics by González-Plaza *et al.* (2017) expressed per 16S rRNA gene copy. Gene product names are placed in parentheses and names of the corresponding active clones are placed in front of the parentheses. Asterisks indicate significance level after correction for multiple testing.

Figure 3.



3.4 Effects on mobile genetic elements

Next, we investigated whether the exposure to macrolide antibiotics had an impact on the composition of mobile genetic elements in the sludge samples. We found that the total abundance of integrases and transposases was significantly higher in the industrial samples ($p = 1.32 \times 10^{-6}$; Figure 4A), consistent with an enrichment of mobile antibiotic resistance genes. Furthermore, the relative abundance of known plasmids was higher in the industrial samples ($p = 1.95 \times 10^{-6}$; Figure 4B). In line with this observation, we could also only recover complete or near-complete plasmids from the industrial libraries. The number of different plasmids detected was also found to be significantly higher in the industrial samples than in the municipal (1497 vs. 926 on average, $p = 0.00067$). The most common resistance genes carried on the detected plasmids were *sul1*, *sul2*, *flo*R, *aph(6)-Id* and *tet(G)*. At the same time, only four MLS resistance genes were associated with these plasmids (*msr(E)*, *mph(E)*, *mph(A)* and *erm(B)*), all of which were carried by a small number of plasmids.

Figure 4.

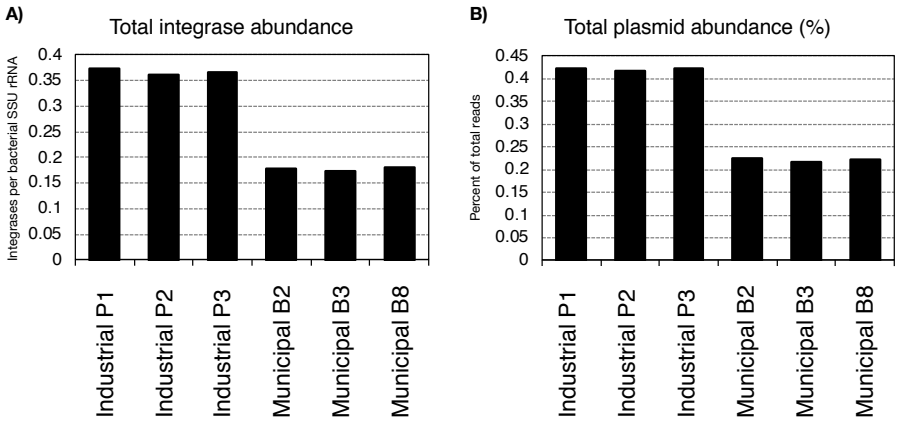


Figure 4. Total abundance of integrases and transposases per 16S rRNA gene copy (A) and percentage of reads mapping to known plasmids (B) in the industrial and municipal sludge samples.

4. Discussion

A key factor in curbing the development of antibiotic resistance in the environment is to limit the number of settings where selection for resistance is likely to occur (Bengtsson-Palme *et al.*, 2018b). WWTPs are well known point sources for the discharge of antibiotics and antibiotic resistant determinants into surface waters (Michael *et al.*, 2013; Rizzo *et al.*, 2013), and therefore critical control points for interventions. Of particular concern are WWTPs that receive wastewaters from pharmaceutical production as they have been discovered to be releasing high levels of antibiotics, often close to therapeutic concentrations (mg/L range). Although most such examples have been described in Asia (Larsson, 2014), the very high levels of antibiotics in treated wastewaters from the Croatian pharmaceutical manufacturing facility investigated here showed that the problem is not isolated to that part of the world (Bielen *et al.*, 2017). Here we describe high, mg/L levels of macrolide antibiotics, particularly azithromycin, in a WWTP processing pharmaceutical wastewater. These concentrations were more than hundred-fold higher than the minimal inhibitory concentrations for some bacterial species, and way above the predicted no-effect concentrations for resistance development (Bengtsson-Palme and Larsson, 2016a), and were accompanied by high levels of a range of antibiotic resistance genes from several different classes.

Interestingly, we did not detect a general accumulation of known MLS resistance genes. Rather, only the second-most abundant macrolide resistance gene – *erm(F)* – showed significantly higher abundance in the industrial compared to municipal samples, while the most abundant gene (i.e. *mphE*) unexpectedly showed lower abundance. Several possible explanations exist for this finding. First, the known MLS resistance genes, such as the *erm*, *msr*, *mef* and *mph* genes, may not provide sufficiently high levels of resistance to withstand the extensive azithromycin exposure in the

348 industrial samples. The *erm* genes encode ribosomal methylases, the *mph* genes encode macrolide
349 phosphotransferases, while the *mef* and *msr* genes encode efflux pumps. It would be reasonable to
350 assume that efflux and/or phosphotransferase activity alone may not be sufficient to detoxify the
351 bacterial cells from azithromycin at the necessary rate to induce resistance at high concentrations.
352 Among the *erm* genes, on the other hand, several showed higher abundance in the industrial
353 samples, although only significantly so for *erm(F)*. This hints at the possibility that ribosomal
354 modification may provide a more efficient resistance mechanism at high concentrations. It also
355 relates to the second possible explanation for the lack of high overall macrolide resistance gene
356 levels; namely that most of the resistance could be due to mutations in the target for the antibiotic
357 – the 23S rRNA gene. We attempted to quantify if there was higher incidence of chromosomal
358 macrolide resistance mutations in the industrial samples, but unfortunately the results were
359 inconclusive due to low numbers of mapped reads. In an earlier study employing functional
360 metagenomics on sediments from the receiving river to explore novel genes providing a resistance
361 phenotype (González-Plaza *et al.*, 2017) we found both known and novel macrolide resistance
362 genes. Surprisingly, we did not detect higher abundances of these genes in the industrial samples
363 compared to those from the municipal WWTP. That said, it cannot be excluded that some yet
364 unknown macrolide resistance genes were present in the industrial sludge samples, which were not
365 detected in our previous study, and therefore not identified in this study either. The community
366 structure was markedly different in the industrial samples, which provides a third possible
367 explanation for the lack of a general macrolide resistance gene augmentation; the extraordinary
368 exposure to antibiotics is likely to have created an environment selecting for species and strains
369 that are intrinsically resistant to azithromycin and erythromycin and therefore do not need to
370 acquire mobile resistance determinants. It should also be noted that the concentrations of

azithromycin, as well as the macrolide resistance gene abundances, were fairly high in the municipal treatment plant compared to previous findings in such environments (Michael *et al.*, 2013; Bengtsson-Palme *et al.*, 2016a; Östman *et al.*, 2017). This could be explained by much higher consumption of antibiotics, including macrolides, in Croatia in comparison with many other European countries (World Health Organization, 2018). Moreover, data on outpatient MLS use in 33 European countries during 1997-2009 showed that the long-acting macrolides, mainly azithromycin, were the most used MLS antibiotic in Croatia (Adrianssens *et al.*, 2011). A consequence of this may be that mobile macrolide resistance genes have already been selected for in the bacteria occupying the municipal sludge and that an additional increase of the azithromycin concentration may have forced chromosomal resistance rather than further acquisition of horizontally transferrable resistance traits. Notably, this type of effect has been observed before in an Indian river subjected to pollution with fluoroquinolones. In that study, the sites with the highest concentrations of ciprofloxacin showed lower levels of mobile fluoroquinolone resistance genes (*qnr*), while less polluted samples harbored high levels of such genes (Kristiansson *et al.*, 2011). Similar results were found in an oxytetracycline production WWTP, where bacteria were more resistant in the effluent compared to the receiving river despite carrying fewer resistance genes (Li *et al.* 2010). These combined findings suggest that at high levels of antibiotic pollution, selection may mainly favor taxonomic shifts towards an intrinsically resistant community or strains harboring resistance mutations, while only extremely efficient mobile resistance genes will be able to provide a selective advantage.

While there did not seem to be noticeably higher overall levels of macrolide resistance genes in the industrial samples, several other types of resistance genes had significantly higher abundances.

These included aminoglycoside, amphenicol, sulfonamide, tetracycline and trimethoprim resistance genes. The majority of the resistance genes enriched were the "usual suspects", i.e. the same genes that have commonly been detected to be enriched in association with antibiotic disturbances. For example, the *sul2*, *aph(6)-Id*, and *strA* genes have often been found co-located on the same mobile genetic element (Sundin and Bender, 1996; Bengtsson-Palme *et al.*, 2016a) and were highly abundant in an Indian lake exposed to antibiotic production waste (Bengtsson-Palme *et al.*, 2014). Similarly, *sul1* was detected at the highest level near a drug formulation facility in Pakistan along with high concentrations of antibiotics (Khan *et al.*, 2013). The *sul2*, *aph(6)-Id*, and *strA* genes were also enriched in the gut microbiome of Swedes returning from travel in Asia or Africa (Bengtsson-Palme *et al.*, 2015a). Furthermore, the *flo*R gene increased in abundance after exposure to ciprofloxacin or tetracycline (Lundström *et al.*, 2016; Kraupner *et al.*, 2018) and *sul1* was enriched in response to tetracycline (Lundström *et al.*, 2016). On the other end, *tet(31)* had 243-fold higher abundance in the industrial samples and is a comparably uncommon resistance gene. None of the known *tet(31)*-carriers were detected in such abundances that it could explain this difference, suggesting that this gene was present in a so far unknown host. The higher abundances of these resistance genes could conceivably be due to selection of specific taxa carrying them. Macrolides are more likely to be effective against gram-positive bacteria, but we did not see lower levels of gram-positive bacterial species. Rather, they had slightly higher abundance overall in the industrial samples. Taken together, the highly mobile nature of the identified genes and the fact that they are not particularly associated with any single host (with the exception of *tet(31)*) suggest that the difference in abundance is caused by antibiotic exposure driving increased genetic mobility.

414 The wide diversity of resistance genes with higher abundance in the industrial samples, the
415 increased abundance of a set of common disturbance-associated genes – many of which are
416 associated with integrons – along with the higher integrase and plasmid abundances suggest that a
417 general feature of high-level antibiotic exposure is that microbial communities respond by
418 mobilizing DNA. This could take the form of horizontal gene transfer between bacteria, increased
419 reshuffling of both plasmids and chromosomal genes, as well as mobilization of genes from
420 chromosomes to plasmids. Interestingly, macrolide antibiotics are not thought to induce the
421 bacterial SOS response (Mo *et al.*, 2016), which is usually attributed to increased rates of horizontal
422 gene transfer in response to stress. Therefore, the increased DNA mobility is likely a result of other
423 stress response pathways or resulting from a longtime selection for bacteria carrying mobile genetic
424 elements. The latter is congruent with what has been argued by Gillings and Stokes, who stipulate
425 that exposure to high concentrations of antibiotics may contribute to an overall increased bacterial
426 evolvability (Gillings and Stokes, 2012; Gillings, 2013). This may also lead to aggregation of novel
427 traits in bacteria, resulting in "superbugs" that are not only resistant to most antibiotics, but also
428 invade more efficiently and are more virulent (Gillings, 2016; Bengtsson-Palme *et al.*, 2018b).
429 Understanding the environments that provide a strong selection pressures from antibiotics is
430 therefore important not only in order to curb the development of antibiotic resistance, but also to
431 comprehend the secondary effects that antibiotic selection may have on bacterial communities
432 beyond selection for resistance. In the context of this study, this is particularly important as the
433 taxonomic diversity of the industrial samples was almost as high as for the municipal samples,
434 suggesting that a wide range of bacteria are able to survive at high concentrations of macrolides.
435 This is also supported by our most recent observations, which indicated that taxonomic diversity
436 of bacterial communities in river sediments highly polluted with macrolides from the same

437 industrial WWTP (up to 24 mg of azithromycin/kg of sediment) was similar to that of bacterial
438 communities in upstream reference sediment (Milaković *et al.*, 2019).

439 This study provides further evidence for the importance of pharmaceutical WWTPs and aquatic
440 environments receiving their polluted wastewaters for the selection of antibiotic resistance. Such
441 polluted matrices host a range of resistance factors and have been shown to be important sources
442 of resistance genes, known as well as novel (González-Plaza *et al.*, 2017; Marathe *et al.*, 2018). The
443 fact that both abundances of mobile genetic elements and resistance genes were higher in the
444 industrial samples raises the concern that those resistance genes may be, or become, mobile and
445 spread to human pathogens, leading to failure of antibiotics treatment in healthcare. While much
446 work has been focusing on increasing the treatment efficiency for sewage, improved management
447 of discharges from antibiotic production may be a more urgent goal in terms of hindering resistance
448 development. One possible solution to this problem would be pretreatment of wastewater from
449 antibiotic production by, e.g., ozonation to reduce the concentrations of antibiotics that the
450 activated sludge is exposed to. Such a solution would decrease the selection pressure for resistance
451 in the activated sludge and at the same time lower the antibiotic concentrations in the treated
452 wastewaters. Discharge management also includes defining emission limits for individual antibiotic
453 substances. Proper emission limits are particularly important for compounds that are shown to
454 pose environmental and/or health risks, such as macrolides, which have high toxicity, persistence
455 and bioaccumulation potential (Bielen *et al.*, 2017; Bengtsson-Palme and Larsson, 2018). Due to
456 these properties, macrolides are included in the EU watchlist for water monitoring (European
457 Commission, 2015). The importance of establishing discharge limits for antibiotics from
458 manufacturing sites has been highlighted before (Review on Antimicrobial Resistance, 2016;

Bengtsson-Palme and Larsson, 2016b; Bielen *et al.*, 2017; González-Plaza *et al.*, 2017; Bengtsson-Palme *et al.*, 2018a; Le Page *et al.*, 2017), but deserves to be emphasized again. It would be easy to write off the problem of environmental pollution with pharmaceuticals as primarily a concern in countries with poor pollution control, since price pressure has led to outsourcing of global antibiotics production to locations with lax environmental regulation (Bengtsson-Palme *et al.*, 2018a). From that perspective, one could get the impression that there would be little incentive for improving legislation regarding emissions from pharmaceutical manufacturing at the EU level. However, this study – together with other studies on European production facilities (Bielen *et al.*, 2017; González-Plaza *et al.*, 2017) – makes clear that regulation is urgently needed, also in Europe.

5. Conclusions

In this paper, we have shown high abundances of antibiotic resistance genes in a wastewater treatment plant in Croatia receiving wastewater from the production of the macrolide antibiotic azithromycin. Remarkably, overall macrolide resistance gene abundances were not higher than they were in a municipal WWTP, while the abundances of resistance genes commonly associated with mobile genetic elements such as integrons were. This suggests that exposure to high levels of antibiotics results in increased genetic mobility in microbial communities. That said, the lack of higher macrolide resistance gene levels leads us to conclude that the strong selection from macrolide antibiotics has favored taxonomic shifts towards intrinsically resistant species – or strains with chromosomal resistance mutations – over the acquisition of mobile resistance determinants to macrolides. The results highlight that there is a need for regulatory action within Europe to avoid releases of antibiotics into the environment.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

The study was conceived by NUK and designed by JBP and NUK. Sample processing was performed by MM and MG. Chemical analysis was done by HS and RG. JBP analyzed data with assistance from NUK. VJ contributed statistical guidance. JBP and NUK drafted the manuscript. All authors read, contributed and approved of the final manuscript.

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