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Antibiotic Resistance in Wastewater and Its Impact on a Receiving River: A Case Study of WWTP Brno-Modřice, Czech Republic

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Abstract: Antibiotic resistance has become a global threat in which the anthropogenically influenced aquatic environment represents not only a reservoir for the spread of antibiotic resistant bacteria (ARB) among humans and animals but also an environment where resistance genes are introduced into natural microbial ecosystems. Wastewater is one of the sources of antibiotic resistance. The aim of this research was the evaluation of wastewater impact on the spread of antibiotic resistance in the water environment. In this study, qPCR was used to detect antibiotic resistance genes (ARGs)—blaCTX-M-15, blaCTX-M-32, ampC, blaTEM, sul1, tetM and mcr-1 and an integron detection primer (intl1). Detection of antibiotic resistant *Escherichia coli* was used as a complement to the observed qPCR results. Our results show that the process of wastewater treatment significantly reduces the abundances of ARGs and ARB. Nevertheless, treated wastewater affects the ARGs and ARB number in the receiving river.

Keywords: antibiotic resistance; wastewater treatment plant; antibiotics; genes coding for antibiotic resistance; antibiotic resistant bacteria; qPCR; WWTPs efficiency

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1. Introduction

Antibiotics are routinely applied both in human and veterinary medicine for the treatment of infectious diseases [1–5]. However, worldwide intensive misuse of antibiotics caused their continuous release into the environment [6–11], and the increase of antibiotic resistant bacteria (ARB) [12–14]. Large numbers of clinical ARB harbor antibiotic resistant genes (ARGs) and genetic elements which can be further transmitted to and among environment bacteria [15–17]. In contrast to many chemical contaminants, bacterial contaminants may persist or even spread in the environment [18]. Increasing exposure of environmental bacteria to antibiotics, ARB and ARGs leads to the rapid development of their resistance and potentially increase in the abundance of resistance genes within the environmental resistance genes pool, aka "the resistome", consequently propagation of antibiotic resistance genes between bacteria [15,17,19,20]. Hence, the effect of antibiotics and ARGs and ARB that is released by humans into the environments is regarded as an important environmental problem and potential risk for human health [18,21,22] (Figure 1).

The development of resistance to antibiotics has been often perceived to be solely related to the misuse of antibiotics [12,13,23]. Currently, antibiotic resistance epicenters are found also in many environments [24], including hospital effluents, wastewater treatment

systems or pharmaceutical effluents [25–27]. These sites are peculiar for an enormous number of bacteria coupled with subclinical concentrations of antibiotics, promoting the release of ARB and ARGs into the surrounding environment [28–30]. The Proliferation of ARB and ARGs occurs via multiple mechanisms: (i) due to selection pressure exerted by antibiotics even at extremely low (subinhibitory) antibiotic concentrations or (ii) due to attaining resistance by horizontal gene transfer (HGT) from other bacteria [31,32]. Conjugation, transformation and transduction are commonly known HGT processes involved in ARB development [33,34], and consequently the spreading of ARGs in the environment [33,35,36]. Resistance genes are usually coupled with mobile genetic elements (MGEs, mobilome) including transposons and integrons and can be transferred between distantly related bacteria corresponding to different phyla [37,38]. However, currently, we do not know to what extent ARGs occur in both human pathogenic bacteria and natural bacteria originating from the same reservoirs [18,39].

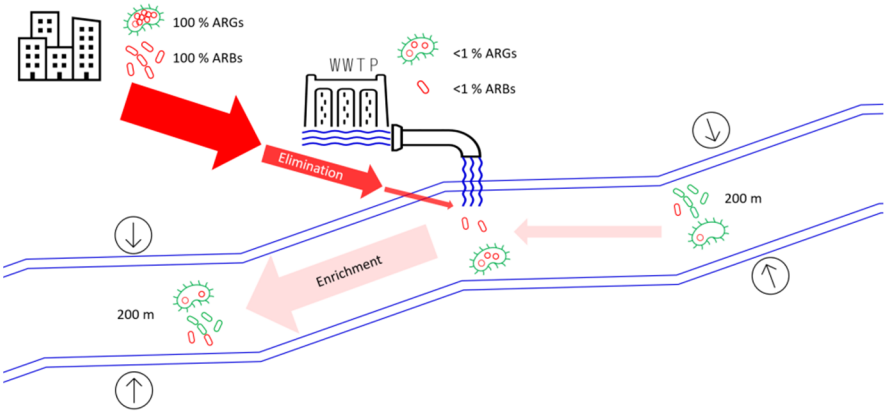

Figure 1. The research focused on estimating the efficiency of wastewater treatment at WWTP Brno has shown high efficiency in the elimination of bacteria and ATB resistance genes in wastewater. Even though the removal efficiency is around 99%, the environment is likely to be enriched. However, according to our results, this enrichment is not already statistically significant 200 m downstream and the values are comparable to upstream.

An immense amount of antibiotics is discharged into wastewater due to imperfect human metabolism and disposal of unused antibiotics [3,40,41]. Wastewater treatment plants (WWTPs) receive sewage from various sources, including hospitals and households, representing important sources of antibiotics [4,26,42], as well ARB and ARGs [43–47] for receiving water bodies [10,11,28,29,48,49]. There is a global consensus that wastewaters belong to the main reservoirs of ARGs [49–52] and sites significant for the proliferation and dissemination of antibiotic resistance [27,53].

Current WWTP technologies are barely able to reduce efficiently or eliminate all microorganisms [54–57]. Rather, the biological wastewater treatment process offers ideal conditions for both bacterial and ARGs proliferation due to nutrients and optimal temperature and enhanced occurrence of horizontal gene transfer [10,58]. Susceptible bacteria are continually in contact with antibiotics at low, sub-inhibitory concentrations, which may impose selective pressure on ARB [4,59]. Various coselection factors such as non-antimicrobial pharmaceuticals) entering WWTPs are responsible for ARB/ARG proliferation [60,61], promoting gene transfer between ARB and susceptible non-ARB [25,47,62]. As a consequence, WWTP effluents represent the most important path for the dissemination of antibiotic resistance to the water environments [27,28].

Although WWTP effluents usually contain a much lower abundance of ARB and ARGs than raw wastewater, researchers have proved that the discharge of treated wastewater may increase the quantity of both ARB and ARGs in the receiving water bodies [63,64]. Moreover, the river stretches downstream of WWTPs can be enriched also with mobile genetic elements [32,65,66], which represent effective carriers of ARGs (including multi-resistances).

However, the mechanisms responsible for the transport, transfer and accumulation of ARGs in river ecosystems remain partially understood. Two hypotheses were proposed to explain these findings: (1) antibiotics released into the environment select the resistant populations, thus increasing the amount of ARGs; (2) ARGs from other sources are routed through runoff processes into the aquatic environment [33]. It is convincing that WWTP effluents may deliver ARGs and mobile elements carrying resistance into downstream aquatic environments [28,29,42,53,67].

Bacteria producing antibiotics occur naturally in the aquatic environment [20,48,66,68,69]; the contact of these environmental bacteria with bacteria from anthropogenic sources provides ideal conditions for the appearance of new resistant strains. Thus, aquatic environments may afford an ideal setting for the exchange of MGEs encoding antibiotic resistance because they are frequently impacted by anthropogenic activities [37,48,70]. Hence, they play an important role in driving the dynamics of antimicrobial resistance in the environment [71]. Upon their entry to the ecosystem, antibiotics may affect the community structure [13] and the activity of environmental microbial populations [72]. Thus, serious worries concerning the potential impacts of antibiotics in the water environment have been already published [19,73,74].

Even though often and abundant presence of both ARB and ARGs in wastewater has attained great interest among scientists and many publications appeared during the last decade [18,25,34,75], there is still a lack of research devoted to this topic in the Czech Republic. Hence, the main objective of this work was to investigate the distribution and characteristics of selected ARB and ARGs in raw and treated wastewater and the removal efficiency of a particular WWTP. In addition, we examined how the discharge of wastewater effluents from the WWTP affects the ARGs and ARB number in the receiving river. Six antibiotic resistance elements which are commonly used as relevant indicators of resistance to various antibiotics classes (e.g., β-lactams, sulfonamides, tetracycline, or colistin) were chosen in our study. These ARGs are commonly found in urban wastewaters and aquatic environments. ARGs encoding a broad spectrum of β-lactamases (genes blaCTX-M and blaTEM) were selected because of their resistance to the basic class of antibiotics used for the treatment of infectious diseases [76]. Gene mcr-1 encodes the resistance to colistin of which the occurrence and prevalence of WWTPs are curious since its detection in treated wastewaters has been proved only sporadically [77,78]. The intl1 gene encoding class 1 integron integrases mediate the capturing of mobile gene cassettes [79]. Moreover, they could be often embedded in promiscuous plasmids and transposons, advancing their lateral transfer [80]. This intl1 gene has been found abundantly both in wastewater and freshwater environments. Some studies suggest that antibiotics like tetracycline, sulfonamides, macrolides, or β-lactams show a significant correlation with the intl1 gene, therefore, it is used as a proxy indicator of anthropogenic pollution [81]. As a complement to ARGs detection, *Escherichia coli* (EC) was chosen as the model microorganism to study phenotypic antibiotic resistance. EC is one of the indicators of fecal contamination in the water environment, which is well described in terms of acquired antibiotic resistance [18]. In our study, EC was examined for resistance to antibiotics corresponding to the above-mentioned ARGs—ampicillin, ceftazidime, cefotaxime, sulfamethoxazole, tetracycline, and colistin.

2. Materials and Methods

2.1. Sampling

Sampling was performed monthly from November 2019 to October 2020. Samples were taken from the influent and effluent of Brno-Modřice WWTP, Czech Republic (population equivalent (PE): 530,000, average flow rate: 1950 L/s) and from the river Svratka where the treated wastewater is discharged. It is a mechanical-biological wastewater treatment plant with a nitrification and denitrification stage and phosphorus removal by simultaneous precipitation. The schema of sampling points is found in Figure 2, Table A1.

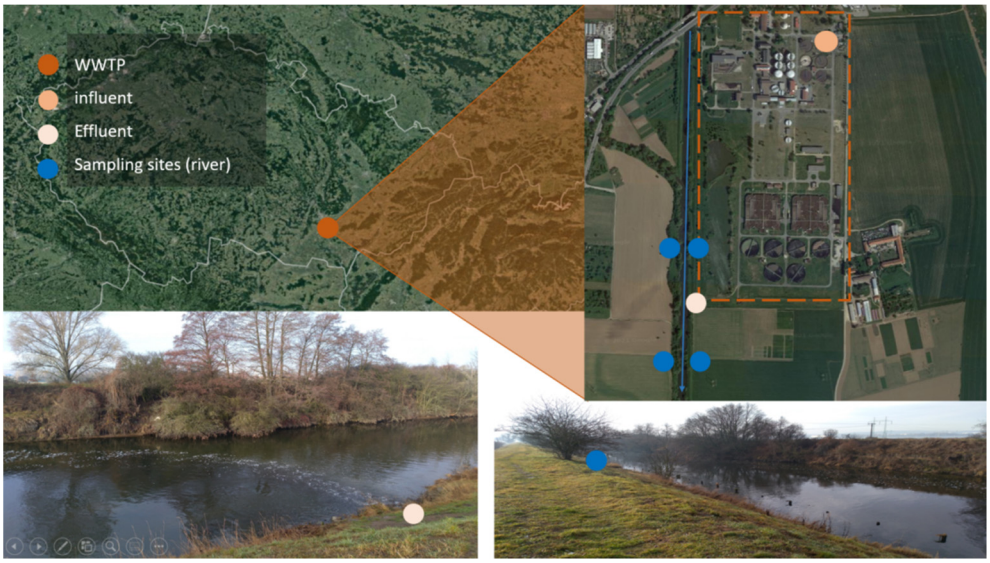

Figure 2. The schema of sampling sites.

There were the following categories of samples:

- Surface water samples;
- River sediment samples;
- Raw and treated wastewater samples;
- Sampling was performed as described by Cacace et al., 2019 [75].

Surface water and river sediment were sampled monthly upstream and downstream of the WWTP at the distance from WWTP approximately 200 m. The water and sediment samples were collected from both the left and right banks and transported in sterile glass bottles or 50 mL plastic falcons (sediment). Sediment samples were taken by hand grab.

The 24h composite samples (flow dependent) of raw and treated wastewater were provided by WWTP staff. Samples of treated wastewater were collected in sterile glass bottles and stored in a fridge during three consecutive days according to Cacace et al., 2019 [75]. Immediately after sampling, the samples were cooled and stored at 5 ± 3 °C until further processed. The analysis was performed within 24 h after sampling.

2.2. Molecular Biology Methods

2.2.1. Sample Processing for PCR Analysis

- Surface Water Samples

Samples were processed according to Cacace et al., 2019 [75]. Briefly, surface water samples from both banks were mixed to form one integrated sample of upstream surface water and one integrated sample of downstream surface water. Three aliquots of 150 mL were filtered through polycarbonate membrane filters (0.22 µm, Isopore Millipore) and the filters were then stored at −20 °C prior to DNA extraction.

- River Sediment Samples

Sediment samples from both banks were mixed to form one integrated sample of upstream sediment and one integrated sample of downstream sediment. DNA isolation followed immediately.

- Raw and Treated Wastewater Samples

Three 150 mL aliquots of treated wastewater and or 50 mL aliquots of raw wastewater samples were filtered through polycarbonate membrane filters (0.22 µm, Isopore Millipore) and the filters were then stored at −20 °C prior to DNA extraction.

2.2.2. DNA Isolation

DNA from water samples was extracted with WaterDNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

DNA from sediment samples was extracted as follows: Samples were centrifuged at 4000 RFC for 5 min and then DNA isolation was done with DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. Isolated DNA was stored at −20 °C prior to qPCR analysis.

2.2.3. Quantitative PCR Analysis

To determine the relative quantity of selected ARGs using qPCR, primers for analysis of ARGs coding for resistance to beta-lactam antibiotics (blaCTX-M-15, blaCTX-M-32, ampC, blaTEM), sulfonamide (sul1), tetracycline (tetM) and polymyxin (mcr-1) were used. In addition, an integron detection primer (intl1), which is responsible for ARGs transfer, and a 16S DNA amplification primer (providing an estimate of the total prokaryotic population in the sample) were used as an internal control. Oligonucleotide sequences and PCR reaction conditions were taken according to Cacace et al., 2019 [75].

qPCR was performed in 20 µL reaction volumes in 96-well plates using LightCycler® Instrument II (Roche, Basel, Switzerland). Each solution contained 10 µL of Luna® Universal qPCR mastermix (New England Biolabs, Ipswich, MA, USA), 0.45 µL of each forward (F) and reverse (R) (stock concentration 10 mM), 4 µL of water. Finally, 5 µL of template DNA or PCR water (for a negative control) was added. Every reaction ran in triplicate.

Conditions of the reaction programs were as follows: 1 cycle (95 °C, 10 min), 40 cycles (95 °C for 15 s and then 60 °C for 30 s with a single acquisition mode at the end), 1 cycle (95 °C for 15 s then 1 min at 60 °C), 1 cycle (from 60 °C to 95 °C with continuous acquisition mode) for melting curve construction. The detection format was SYBR GREEN I/HRM Dye and data were analyzed via Lightcycler® 480 II. For calculating the relative abundance and changes of the analyzed ARGs, the dCt and ddCt method, respectively, was used [82].

2.3. Cultivation Techniques

2.3.1. Determination of Antibiotic Resistant *Escherichia coli* (AR-EC)

AR-EC was determined by cultivation on media containing selected antibiotics (ATB) using the modified ISO Standards cultivation method [83]. ATB and their concentrations which were used in this assay are listed below (Table 1). ATBs were chosen to correspond to the selected ARGs. Concentrations of ATBs in the cultivation medium were derived from the minimal inhibitory concentration (MIC) indicated by EUCAST (European Committee on Antimicrobial Susceptibility Testing).

Table 1. Antibiotics and their concentrations used in cultivation assays.

ATB		MIC (mg/L)
ampicillin	AMP	8
ceftazidime	CAZ	4
cefotaxime	CTX	2
sulfamethoxazole	SXT	512
tetracycline	TCY	16
colistin	COL	2

2.3.2. Surface Water Samples, Wastewater Samples

Undiluted and diluted water samples were filtered through the membrane filters (0.45 µm, GN-6 Metricel® MCE Membrane Disc Filters, Pall, USA), then the filter was placed on ECC ChromoSelect Selective Agar (Sigma-Aldrich, USA) containing ATB. Cultivation for 24 h at 36 ± 0.5 °C followed. After cultivation typical blue colonies were counted as *Escherichia coli*.

2.3.3. River Sediment Samples

Sediment samples were processed as described by Matějů et al., 2008 [84]. To 10 g of mixed sediment sample, 90 mL saline solution (0.85% NaCl solution) was added. The suspension was homogenized for 15 min. After 5 min in the still position, 1 mL of the suspension was diluted and inoculated on ECC ChromoSelect Selective Agar (Sigma-Aldrich, USA) containing ATB. Cultivation was performed for 24 h at 36 ± 0.5 °C. After cultivation, typical blue colonies were counted as *Escherichia coli*.

2.4. Data Presentation

Relative abundances of ARGs were calculated by *the delta-Ct* method and *delta-delta Ct* method, using data normalization of ARGs copies to 16s rRNA copies, in triplicates from each monthly sample. For data presentation, the standard box plot diagram was used, displaying the median (horizontal line in the box), the lower and upper quartiles (bottom and top box lines), the 10th and 90th percentiles (bottom and top whiskers), and the outliers (circles). Wilcoxon test [85] was used to identify the significant differences in the abundances of ARGs and AR-EC between samples taken upstream and downstream of Brno-Modřice WWTP. Kruskal-Wallis test [86] and Dunn's test [87] were used to identify the significant differences both in the relative abundance of ARGs and in the relative abundance of AR-EC. Statistical tests were performed in R software (version 3.6.0, www.rproject.org).

3. Results

3.1. Antibiotic Resistance Genes and Culturable Antibiotic Resistant Escherichia coli in Wastewater

Generally, all selected ARGs were detected both in influent and treated effluent of WWTP. Relative abundance (median of normalised expression level) of the ARGs in the influent was in order: intl1 > sul1 > blaTEM > tetM > blaCTX-M-32 > blaCTX-M-15 > mcr-1 > ampC. The values of the relative abundance (obtained from qPCR Ct values) of ARGs in the effluent were significantly lower compared to those values from the influent wastewater, indicating the efficient removal of ARGs during wastewater treatment processes (Figure 3). The removal efficiency of individual ARGs varied, the highest efficiency in ARGs removal was found for mcr-1, while the lowest removal efficiency was observed in the case of ampC (Figure 4).

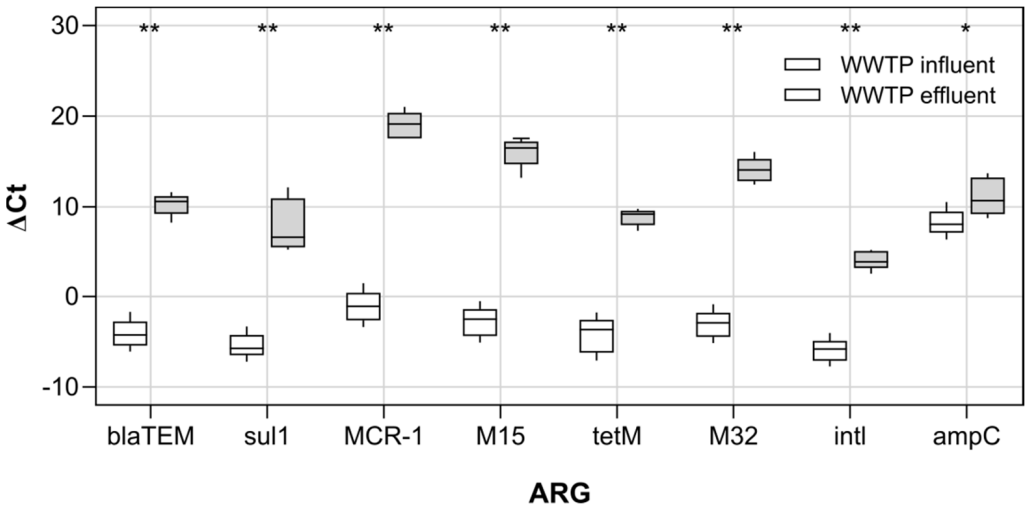

Figure 3. Relative abundance obtained from qPCR Ct values (normalized ARGs copies to 16s rRNA copies) of the seven ARGs analyzed in bacteria from the influent and effluent of Brno-Modřice WWTP. Box plots represent the median (horizontal line in the box), the lower and upper quartiles (bottom and top box lines), and the lower and upper 1.5 IQR (bottom and top whiskers). The statistical difference between the influent and effluent was tested by Mann-Whitney test and the significance is marked with * for $p < 0.05$, ** for $p < 0.01$.

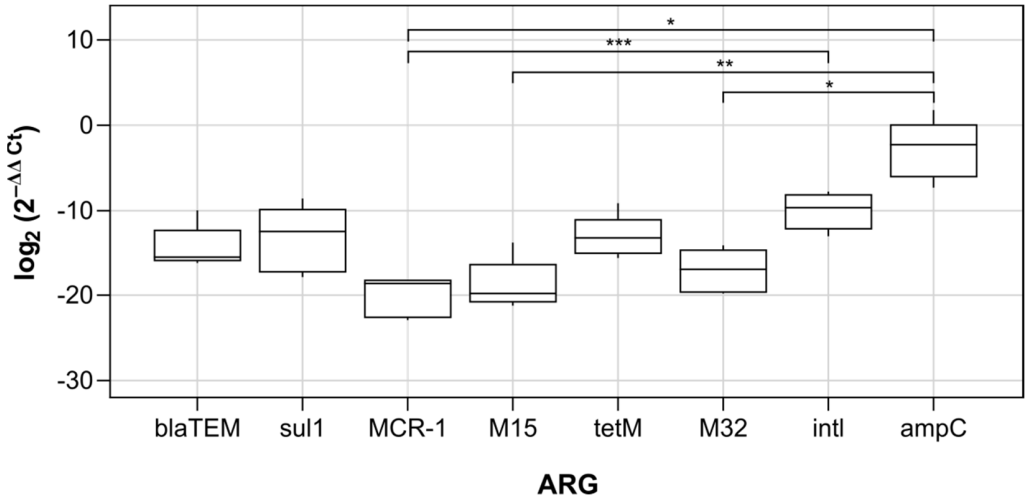

Figure 4. The difference in fold gene expression (normalized ARGs copies to 16s rRNA copies) of the ARGs analyzed between the influent and effluent of Brno-Modřice WWTP. The statistical difference between the groups was tested by the Kruskal-Wallis test and with Dunn's multiple comparisons, and the significance is marked with * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Resistance rate of *Escherichia coli* (percentage of culturable AR-EC to the total culturable EC in a sample) ranged from 51.4% (±16.3%) in raw wastewater to 33.7% (±9.7%) in treated wastewater. The resistance rate in the influent was on average 1.5 times higher than that in the effluent. Unlike ARGs, the highest density in the influent wastewater was found for culturable AR-EC bearing resistance to AMP, followed by SXT, TCY, CTX, CAZ and COL. The AR-EC abundance varied from 10^2 to 10^4 CFU/mL in the influent wastewater and up to 10^2 CFU/mL in the effluent water (Figure 5). The absolute abundance of AR-EC was significantly reduced during the wastewater treatment process (Figure 3). On average, the 99.1% (±0.6%), i.e., 2.22 log reduction in the abundance of AR-EC was found. In the influent/effluent ratios of the abundance of culturable AR-EC in wastewater, statistically significant differences were not found (Figure 6).

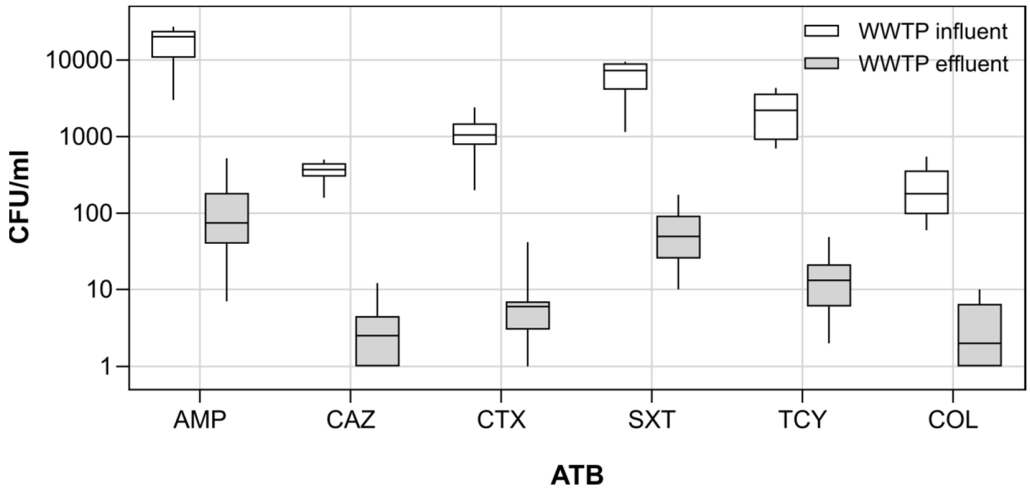

Figure 5. Box plots of the abundance of culturable *Escherichia coli* resistant to ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), sulfamethoxazole (SXT), tetracycline (TCY) and colistin (COL) measured in the wastewater entering WWTP and treated effluents from WWTP.

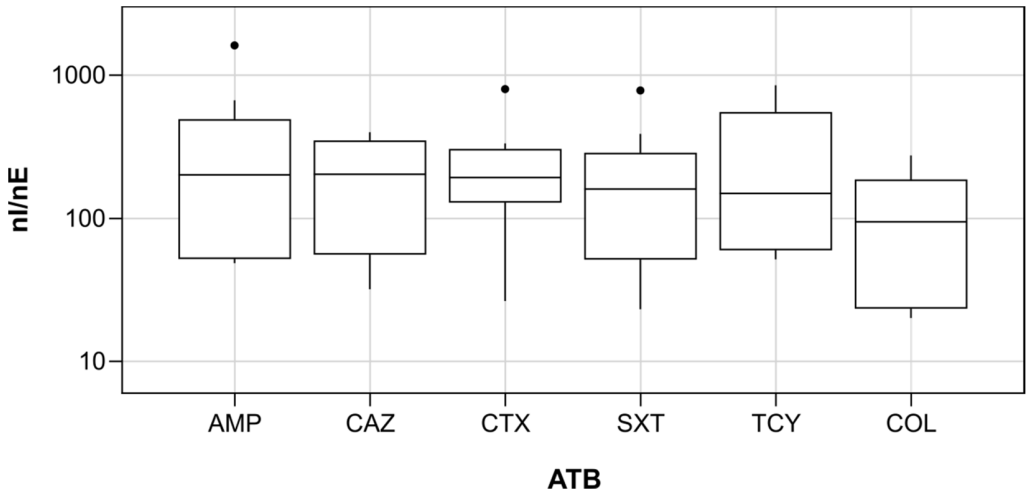

Figure 6. Influent/effluent ratios (nI/nE) of the abundance of culturable *Escherichia coli* resistant resistant to ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), sulfamethoxazole (SXT), tetracycline (TCY) and colistin (COL) measured in the wastewater. • indicate data points located outside whiskers of the Tukey's boxplot (outliers).

3.2. Antibiotic Resistance Genes and Antibiotic Resistant Escherichia coli in River Recipient

Generally, the values of relative abundance of ARGs in river water downstream of the Brno-Modřice WWTP were higher than the values measured at the upstream sites of the Svratka River (Figure 7). Most of the detected ARGs showed the positive ratio of downstream to upstream abundance (nWD/nWU), while the negative ratio was found for MCR- 1 and M15 genes (Figure 8). However, no ARGs showed a statistically significant difference between both the upstream and downstream parts of the river.

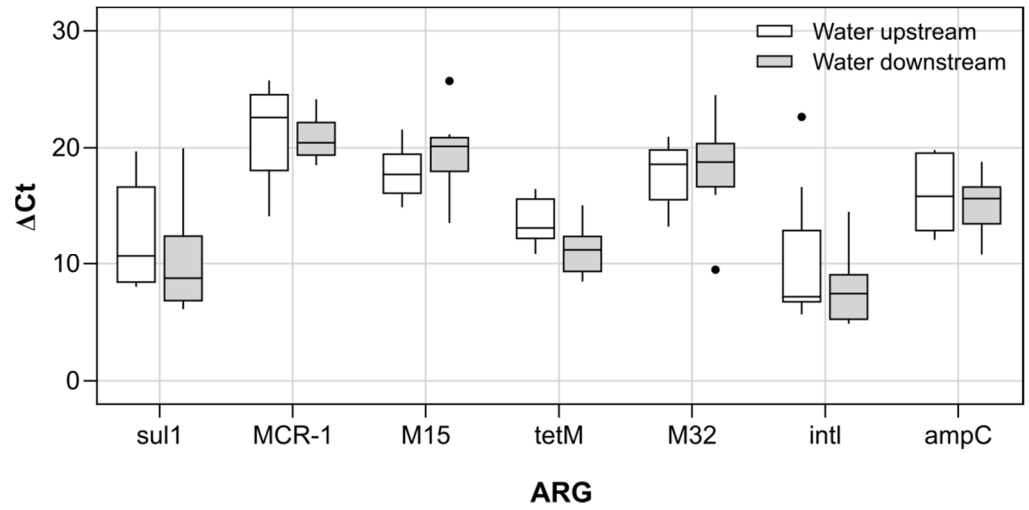

Figure 7. Relative abundance obtained from qPCR Ct values (normalized ARGs copies to 16s rRNA copies) of the seven ARGs analyzed in the surface water taken upstream and downstream of the WWTP analyzed in the surface water taken upstream and downstream of the WWTP. • indicate data points located outside whiskers of the Tukey's boxplot (outliers).

This suggests that despite a significant reduction in the ARGs during the treatment (Table 2), the river water downstream of WWTP was probably slightly (but not statistically significantly) enriched by ARGs released into the environment by the treated effluent.

The abundance of antibiotic resistant *Escherichia coli* in the river water downstream of the WWTP was always higher compared to that AR-EC from the upstream part of the river (Figure 9). *Escherichia coli* resistant to SXT and TCY showed the highest ratio

of downstream to upstream abundances (nWD/nWU), while the lowest ratio was found for *Escherichia coli* resistant to CTX and COL (Figure 10). The abundance of AR-EC in the Svratka River increased on average about 4.5 times from upstream to downstream of the WWTP discharge point to the river (Figure 8), however, statistically significant differences between the abundance of AR-EC upstream and downstream of the WWTP discharge were not found. The resistance rate of *Escherichia coli* ranged from 20% (±5.6%) in river water upstream of the WWTP to 34% (±7.2%) in the river water downstream of the WWTP.

Table 2. Estimated ARGs removal efficiency during wastewater treatment in WWTP Brno Modřice.

Efficiency (%)	blaTEM	sul1	MCR-1	M15	tetM	M32	intl	ampC
Mean	99.76	99.46	99.99	99.96	99.61	99.95	98.73	76.06
Std. Dev.	0.38	0.72	0.01	0.07	0.55	0.05	1.01	20.91

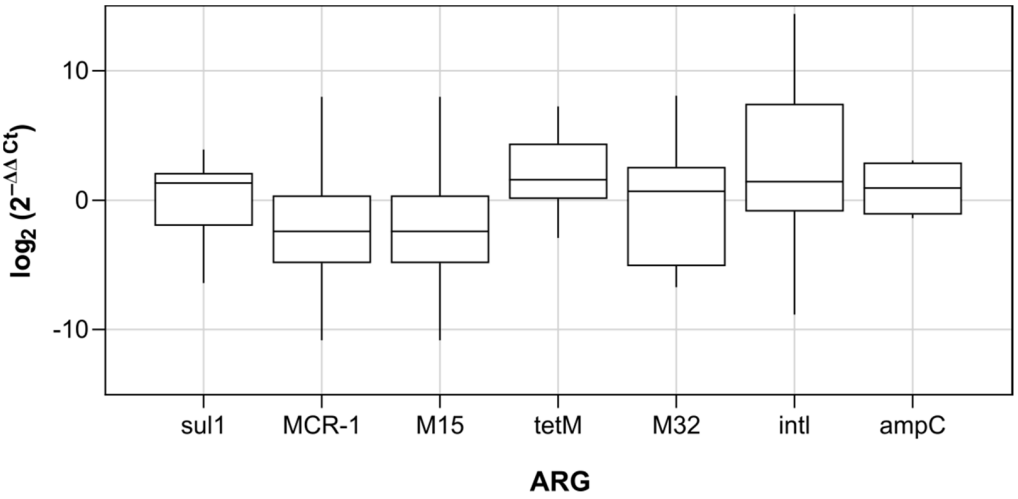

Figure 8. The difference in normalized fold gene expression level of the ARGs between the surface water taken upstream and downstream of the WWTP.

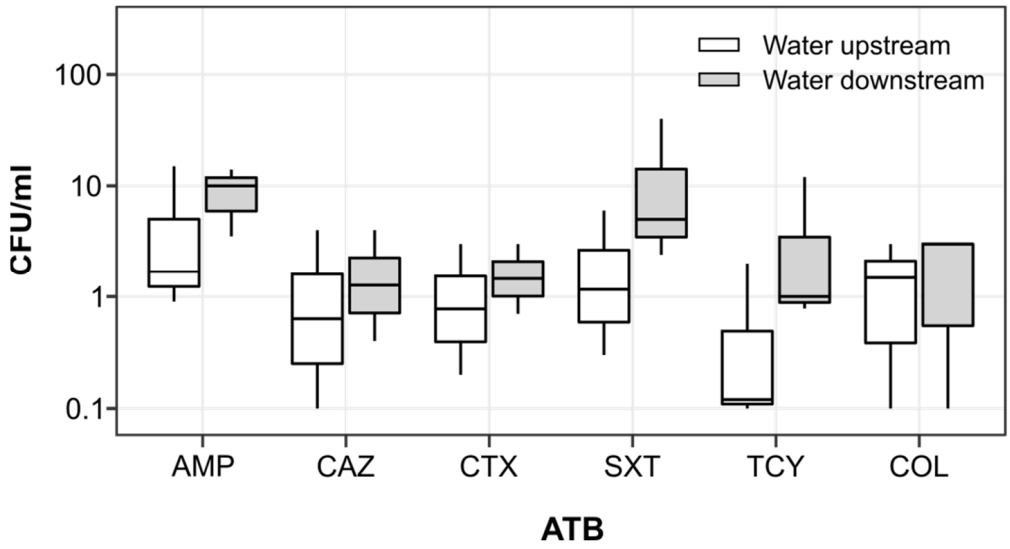

Figure 9. Box plots of the abundance of culturable *Escherichia coli* resistant to ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), sulfamethoxazole (SXT), tetracycline (TCY), and colistin (COL) measured in the river water.

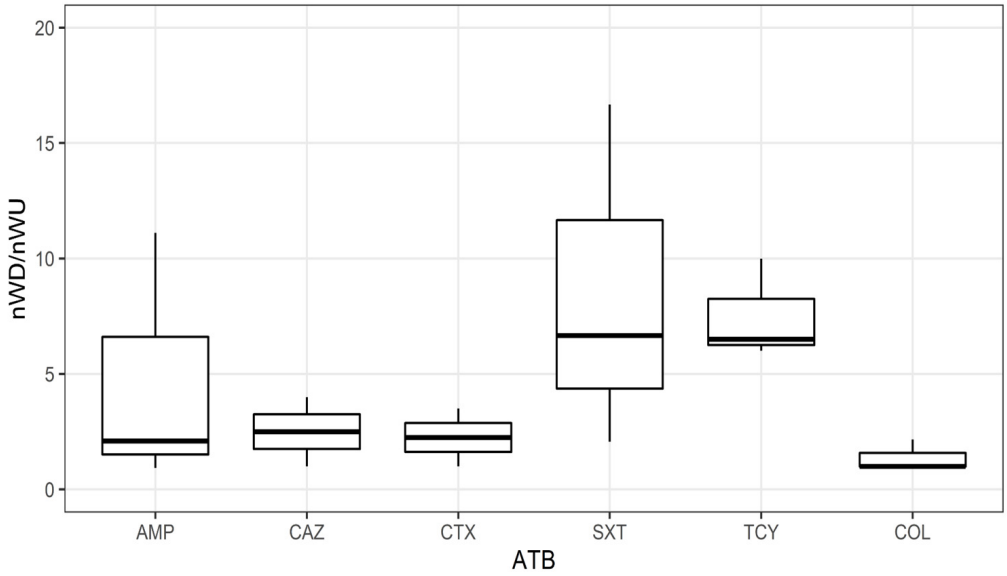

Figure 10. Downstream/upstream ratios (nWD/nWU) of the abundance of culturable *Escherichia coli* resistant to ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), sulfamethoxazole (SXT), tetracycline (TCY) and colistin (COL) measured in the river water.

3.3. Antibiotic Resistance Genes and Resistant Escherichia coli in River Sediments

The values of the relative abundance of ARGs in the surface sediments show much smaller and nonsignificant differences in ARG concentration between upstream and downstream of the WWTP discharge than the values obtained from water samples (Figure 11). Nevertheless, the potential indications of a trace enrichment of the sediments taken below WWTP discharge point were found only in three ARGs (Figure 12).

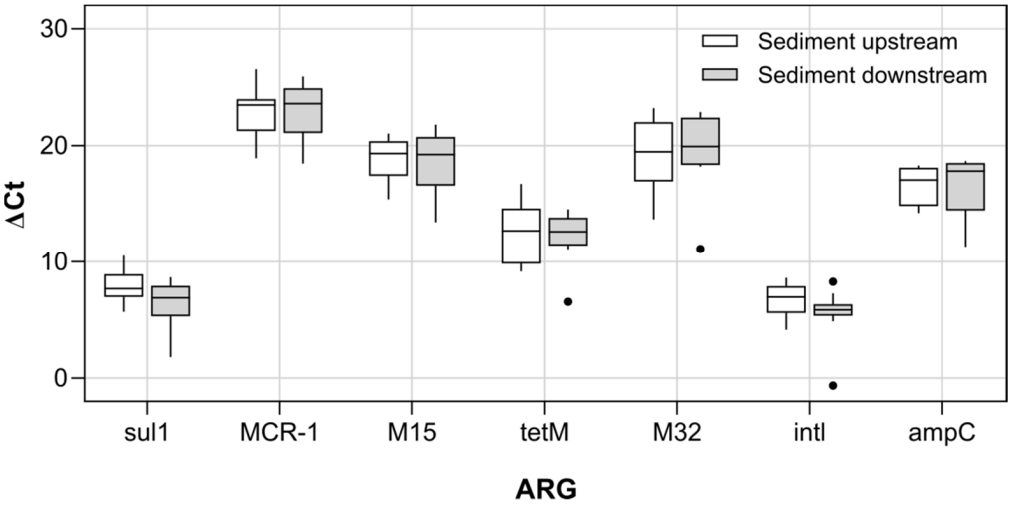

Figure 11. Normalized expression levels of ARGs in the sediment samples taken upstream and downstream of the WWTP. • indicate data points located outside whiskers of the Tukey's boxplot (outliers).

The absolute abundance of AR-EC was found also to be higher in the river sediments below the WWTP discharge (Figure 13). Higher downstream/upstream ratios were observed for *Escherichia coli* resistant to TCY and SXT, while the lowest for *Escherichia coli* resistant to CTX and COL (Figure 14). Nevertheless, compared to the abundance of AR-EC in river water, the ratios between a downstream and upstream part of river sediments reached higher values (Figure 14). The abundance of AR-EC in the Svratka River sediments increased on average about 7.4 times from upstream to downstream of the WWTP

discharge point to the river (Figure 14), however, we found no statistically significant differences between the abundance of AR-EC upstream and downstream of the WWTP discharge. Resistance rate of *Escherichia coli* in the river sediments upstream of the WWTP showed the same value 25% (±7.3%) as samples of the river sediments taken downstream of the WWTP (±7.4%).

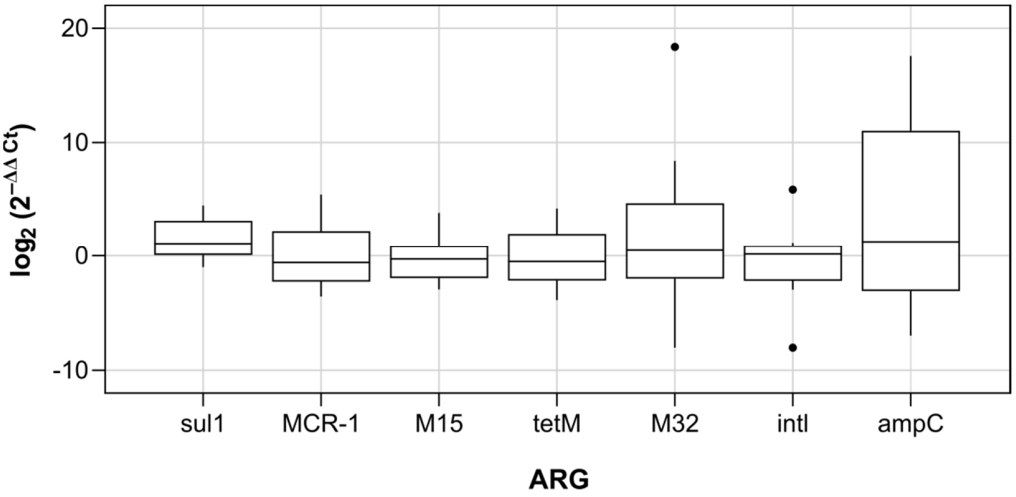

Figure 12. Normalized fold gene expression levels of ARGs in the sediment samples taken upstream and downstream of the WWTP. • indicate data points located outside whiskers of the Tukey's boxplot (outliers).

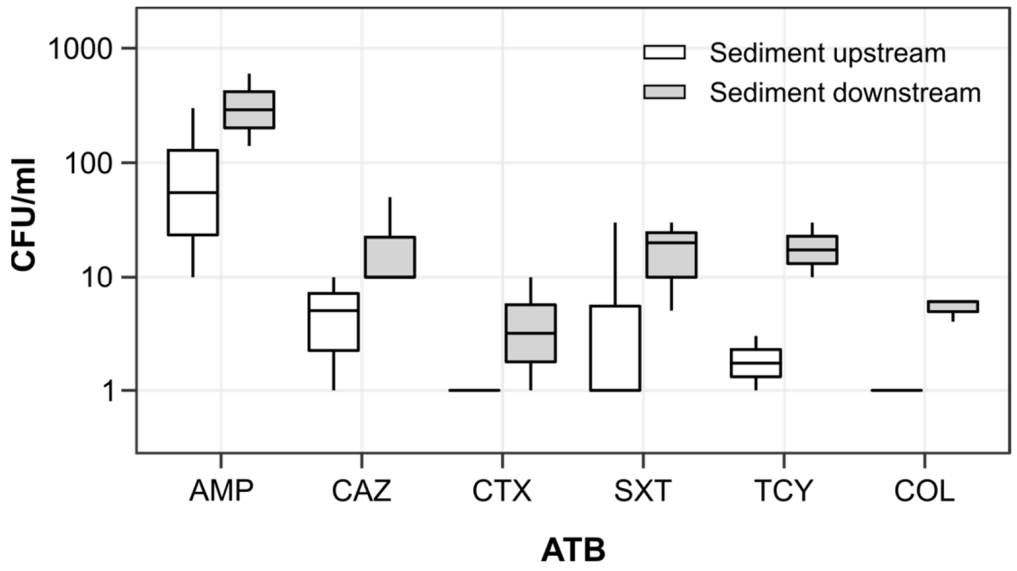

Figure 13. Box plots of the abundance of culturable *Escherichia coli* resistant to ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), sulfamethoxazole (SXT), tetracycline (TCY) and colistin (COL) measured in the river sediments.

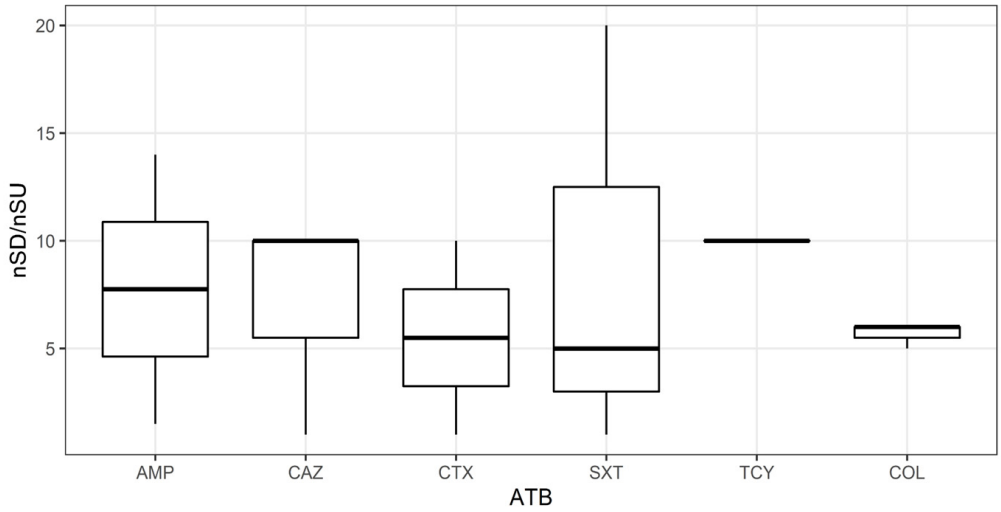

Figure 14. Downstream/upstream ratios (nSD/nSU) of the abundance of culturable *Escherichia coli* resistant to ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), sulfamethoxazole (SXT), tetracycline (TCY) and colistin (COL) measured in the river sediments.

4. Discussion

4.1. Antibiotic Resistance Genes and Antibiotic Resistant Escherichia coli in the WWTP

The most abundant genes in Brno-Modřice WWTP were the class 1 integron integrase gene intl1, the sul1 gene coding for sulfonamide resistance, blaTEM and tetM. These genes intl1 and sul1 have been detected in wastewater treatment plants and in surface waters receiving treated effluents [80,88]. Our finding confirms the results of many published studies [46,47,56,75].

The use of biological treatment processes (activated sludge) to treat antibiotic–containing wastewater raises the question whether use of ARGs and ARB might be multiplied during these processes [27,42,71]. Generally, higher antibiotic residues in WWTPs may significantly affect the fate of ARGs in effluents from WWTP. However, some ARGs showed positive correlations with a residual concentration of antibiotics, but some negative or no significant correlations [89,90]. Hence, the high antibiotic residues in treated wastewater may influence the proliferation and fate of ARGs and ARB in the effluents and consequently their fate in the receiving river. In this study, however, the concentration of neither antibiotic was measured in raw wastewater, nor in WWTP effluent, so we cannot evaluate the potential significance of the antibiotics on the abundance of both ARGs and ARB in the effluent of a Brno-Modřice WWTP.

Nevertheless, our results indicate that the relative abundances of ARGs and the absolute abundance of AR-EC were efficiently reduced during the treatment processes in Brno-Modřice WWTP. This finding is congruent with other studies investigating the fate of ARGs through wastewater treatment [28,56]. Moreover, no proliferation of ARGs or significant augmentation in the resistance rate of *Escherichia coli* was observed during sewage treatment processes. The causes for the increased abundance of ARGs and ARB are not well understood [91].

Although one man expects close relationships between ARGs and AR-EC concentrations [92], it is rather difficult to determine this relationship in real wastewater samples. The main reason is the fact that some ARGs may occur either as intracellular elements inside the bacterial cells (i.e., as a part of intracellular DNA), while some of them as free extracellular DNA. Since the method we used for the detection of ARGs in our samples was based on filtration and extraction of bacterial cell DNA, we have no idea about how much proportion of free ARGs occurred in the surrounding wastewater. Previous unpublished experiments of our colleagues suggest that the ratio of extracellular DNA to intracellular DNA may vary from 1:4 up to 1:12 depending on the type of water (clear natural water vs treated wastewater) or the time of sampling, for instance.

In the case of ARGs, it appears that tetM exhibited higher removal efficiency, while the reduction of sul1 was lower [56]. In this study, the highest efficiency in ARGs removal was found for mcr-1 gene. This ARG, located on highly mobile plasmids, has been reported in numerous papers regarding pig farms and slaughterhouses [93,94], while the rare occurrence of mcr-1 in freshwaters [95,96] might be explained by the relatively high removal efficiency during WWTP processes. Nevertheless, this ARG is able to survive the sewage water treatment process and potentially be persistent also in river recipients [97]. Our data support this suggestion, in spite of the fact that mcr-1 evinced the lowest relative abundance of all observed ARGs both in river water and sediments.

The absolute abundance of *E. coli* resistant to the different antibiotics was significantly reduced in WWTP Brno-Modřice too. This result is also in agreement with that found in other studies [98,99]. On the other hand, the percentage of AR-EC (resistance rate) was reduced throughout the treatment process, while some studies observed invariable or enhanced percentages of AR-EC in WWTP effluents in comparison to the WWTP influents [99–101].

The removal efficiency of ARGs by primary treatment processes is reported to be negligible, however, it seems that most ARGs could be reduced effectively by the activated sludge process [56]. Brno-Modřice WWTP employs traditional treatment processes of primary sedimentation and biological treatment, hence we can attribute the high removal efficiency of both AR-EC and ARGs to these various treatment processes. Nevertheless, there is still up to 10^2 CFU/mL AR-EC (i.e., 0.9% of AR-EC found in influent) and a trace amount of ARGs in the WWPT effluent. For instance, we found that the absolute abundances of AR-EC in the effluent were much higher than those measured at the upstream sites in the Svratka River. As a consequence, the abundance of AR-EC in the Svratka River increased on average about 4.5 times from upstream to the downstream site of the WWTP, suggesting that despite the reduction of total AR-EC during the wastewater treatment process, the discharge of effluents from WWTP contaminated with ARGs and AR-EC poses a high risk of dissemination of those elements into the environment, besides other things because of large amount discharged into the river recipient per day [27,28,63,64].

4.2. Effect of WWTP Effluent on River Downstream Environment

Despite the significant reduction of ARG and AR-EC abundances, Brno-Modřice WWTP treated effluents contain still abundances of ARGs and AR-EC that are higher in both the relative and absolute abundances than those measured in the receiving river. Consequently, the abundance of both ARGs and AR-EC increased, at least in the case of some genes significantly downstream of the WWTP discharge into the Svratka River. Our observations agree with previously published reports that WWTPs can promote and provide conducive conditions for the establishment and spreading of ARB in the receiving river environment [71,102,103].

We found the increased concentration of ARGs and ARB in both the river water and sediment collected downstream of the WWTP discharge point. While detection of ARGs and ARB in river compartments downstream of the WWTP discharge point has been rather expected, the detectable levels of all analyzed ARGs and ARB found in the upstream samples suggest that some antibiotic resistance may naturally occur also in the river environment. Several factors may be responsible for the maintenance of this background resistance in the samples collected at the upstream site, including agricultural runoff and soil leaching [104]. In the case of the Svratka River, we assume that the ARGs and ARB found upstream of Brno-Modřice WWTP discharge point become most likely from a University Hospital WWTP effluent.

ARGs and ARB have been reported to be ubiquitous both in river water and sediments or biofilms downstream of WWTPs [29,68,104,105]. The ARGs in the water and sediment can persist far downstream of the WWTPs [104], even until 20 km downstream from the WWTP effluent discharge point [30], suggesting that some ARGs may persist in the river environment. In our study, genes intl1 and sul1 were found to be the most abundant in river

water and sediments. Despite both genes being efficiently removed during wastewater treatment, their relative abundance in the WWTP effluent remained still too high, causing their spread into the river environment. This observation is consistent with previous studies [29,46,104]. The sul1 abundance was also the highest in groundwater samples [29]. Sediments may serve as a pool of both the ARB and ARGs [68]. Our findings support this hypothesis, particularly concerning the behavior of AR-EC. In comparison with the abundance of AR-EC in river water, the downstream/upstream ratios of abundance in river sediments showed higher values, suggesting that the sediments were more enriched by AR-EC than surface water. Although the values of ARGs found in the effluent are richer compared to river water, about 250 m downstream, the difference between the abundance above and below the WWTP discharge was no longer significant.

5. Conclusions

In conclusion, our data, in congruence with other published studies, show that WWTP effluents may be a source of ARGs and ARB, whenever the wastewater effluent is discharged into a river. Persistence and enrichment of both ARGs and ARB in river water and, namely in river sediments downstream of the WWTP suggest that these antibiotic elements are disseminated and can potentially spread further in aquatic environments, although ARGs amount downstream appears to be reduced spontaneously by natural processes. In the future, we recommend studying river water, sediments and hyporheic interstitial water simultaneously at several distances downstream of the WWTP discharge points to evaluate properly the fate of the antibiotic resistance in the river environment.

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Appendix A

Table A1. Table of sampling points with GPS coordinates.

Sampling Point	Bank	GPS
Svratka-upstream	left	49.1262797N, 16.6270903E
Svratka-upstream	right	49.1262658N, 16.6267364E
Svratka-downstream	left	49.1225339N, 16.6268811E
Svratka-downstream	right	49.1225411N, 16.6265378E
WWTP outflow		49.1244719N, 16.6269778E

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