Determination of antibiotic resistance genes in a WWTP-impacted river in surface water, sediment, and biofilm: Influence of seasonality and water quality

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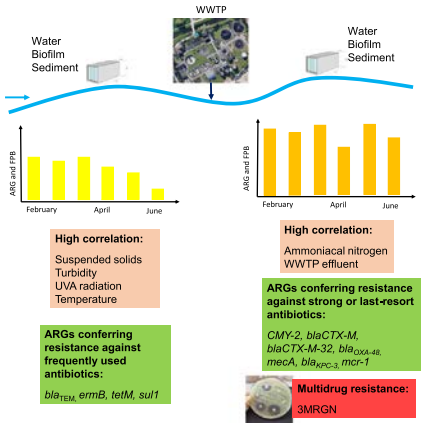
HIGHLIGHTS

- There is a background contamination of *bla*$_{TEM}$, *ermB*, *tetM*, and *sul1*in the basin.
- Multidrug and last resort drugs resis tance were detected downstream of the WWTP.
- The presence of ARG/FPB downstream of the WWTP was influenced by the ef fluent.
- The presence of ARG/FPB upstream of the WWTP was influenced by seasonal parameters.
- The biofilm sampler is an efficient way to collect biofilms from determined periods.

GRAPHICAL ABSTRACT



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ABSTRACT

Many pathogenic bacteria are adapted to live in aquatic habitats, which makes rivers possible sources and spread pathways of antibiotic resistance, since they usually receive effluents from wastewater treatment plants (WWTP), possibly containing antibiotic residues and also antibiotic resistant bacteria. This study investigates dif ferent monitoring strategies to identify the occurrence of antibiotic resistant bacteria in rivers. We analyzed the presence of 13 antibiotic resistance genes (ARGs) and seven gene markers for facultative pathogenic bacteria (FPB) with qPCR in sampling sites upstream and downstream of a small WWTP in Southern Germany. Five sam pling campaigns were conducted from February to June 2019. Surface water, sediment, and biofilm samples were analyzed. The biofilm was collected from an artificial sampler placed in the river. *bla*$_{TEM}$, *ermB*, *tet*M, and *sul*1 genes were detected in all samples analyzed. The results showed there was a previous background in the river, but the WWTP and the water quality of the river influenced the concentration and occurrence of ARGs and FPB. Genes representing resistance against strong or last resort antibiotics, such as *mec*A, bla$_{CMY\ 2}$, *bla*$_{KPC\ 3}$, and *mcr 1*, and multidrug resistance were also detected, mainly in samples collected downstream of the WWTP. Downstream of the WWTP, the occurrence of ARG and FPB correlated with ammoniacal nitrogen, while upstream

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of the WWTP correlated with turbidity, suspended solids, and seasonal factors such as UVA radiation and the presence of macrophytes. Biofilm samples presented higher abundances of ARGs and FPB. The biofilm sampler was efficient and allowed to collect biofilms from specific periods, which helped to identify seasonal patterns.

1. Introduction

Antibiotics mark the beginning of modern medicine. Several diseases have become treatable after the discovery of these substances and their use allowed to perform complex procedures, such as surgeries and che motherapy (Friedman et al., 2016). Antibiotics were discovered from se cretions of microorganisms, including fungi and bacteria themselves. The production of substances with such properties was a defense mechanism against other bacteria, and so is the ability to resist antibiotics (Friedman et al., 2016; Martínez, 2012). After several decades of extensive antibiotic use, resistant bacteria have become more and more frequent.

Bacteria can also develop or acquire resistance against multiple anti biotics and the number of multidrug resistant bacteria is increasing fast (Frieri et al., 2017). In former times, if treatment failed with one specific antibiotic, other antibiotics could be employed. Today, the health com munity is struggling to find efficient treatments for infections because of multiple antibiotic resistance, increasing the consumption of antibi otics (Macgowan and Macnaughton, 2017). This can cause negative consequences for the population, both on individual and public levels.

Since this is a matter of public safety and health, the consumption of antibiotics is monitored in European countries by the European Centre for Disease Prevention and Control (ECDC). Germany, for example, con sumes around 11.9 DDD (defined daily dose) of antibacterials per 1000 inhabitants per day. With these numbers, Germany is the 7th lowest an tibiotics consuming country in Europe. The main antibiotics consumed in the country are penicillin (31.5%), followed by other β lactams (20.9%), macrolides, lincosamides, and streptogramins (16.4%), tetracy clines (13.8%), quinolones (8.1%), and sulfonamides/trimethoprim (5.3%) (ECDC, 2019 Report).

The ECDC also monitors the presence of antibiotic resistance in Europe. While the occurrence of antibiotic resistance in some organisms is not a public health problem, in others, such as in facultative patho genic bacteria (FPB), it is highly concerning. The group of concern in cludes the following species: *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii*, *Streptoccocus pneumoniae* and enterococci (ECDC, 2018). The identification of antibi otic resistance in these microorganisms is very important, since they frequently cause human and animal infections and are easily detected in medical facilities. Some of these species are also adapted to live in other habitats, such as aquatic ecosystems.

Even if most of the concern about antibiotic resistance is towards pathogenic bacteria detected in medical facilities, patients, and infec tions, it is still necessary to pay attention to the growth of antibiotic re sistance in other environments. Regarding this issue, surveillance of resistant bacteria in animals, food, and even in wastewater effluents is advancing fast, but monitoring efforts in the natural environment are still rudimentary and should be improved (Huijbers et al., 2019). The main reason to study antibiotic resistance in the natural environment, mainly in rivers and water bodies, is to better understand the threat to animal and human health, to detected possible reservoirs of resistance and to develop new techniques to prevent the spread and increase of antibiotic resistant bacteria (Bengtsson Palme et al., 2018; Von Wintersdorff et al., 2016). Also, there is a direct link from the natural en vironment (mainly aquatic ecosystems), to human needs, such as the consumption of potable water, irrigation of crops and different kinds of plantations, and the direct consumption of fish and other organisms.

In urban rivers, the effluents of wastewater treatment plants (WWTP) and raw sewage are considered the main sources of antibiotic resistance genes (ARG) (Auguet et al., 2017; Bengtsson Palme et al., 2018; Hembach et al., 2019). Resistant bacteria and ARG have already been detected in surface water, attached to suspended solids (Proia et al., 2018), in the sediment (Brown et al., 2019; Lopes et al., 2016) and in natural biofilms (Guo et al., 2018; Proia et al., 2016; Subirats et al., 2017b).

In rivers and streams, biofilms are attached to pebbles, tree branches, leaves, and even sediment. They are in constant contact with the nutrients, pollutants, and the microbiota present in the water, and have the ability to absorb substances and other microorgan isms, incorporating them into their matrices (Battin et al., 2016; Bechtold et al., 2012). According to these features, biofilm seems to be a suitable medium for detecting antibiotic resistant bacteria and the spread of antibiotic resistance within a natural ecosystem (Balcázarr et al., 2015).

The goal of this research, therefore, was to investigate different monitoring strategies to detect and quantify ARGs and FPB in aquatic compartments. In addition, multi drug resistance was analyzed. Surface water (grab sampling), sediment (core sampling), and biofilm (passive sampling) were collected in a river impacted by human activities in Southern Germany. The influence of physical and chemical parameters such as nutrients, water turbidity, dissolved oxygen, and other external parameters, such as UV radiation, on the occurrence of antibiotic resistance were also evaluated.

2. Experimental design

2.1. Study area

The Kraichbach River is located in Southern Germany, in the state of Baden Württemberg. In this area, the mean temperature reaches 0 °C in January and 18 °C in July. The river is approximately 60 km long with a catchment area of 161 km^2. The lower part of the Kraichbach River is classified as a "heavily modified water body", is strongly rectified with reduced morphological structures, and has a slow flow. The Kraichbach River is the effluent receptor of six WWTPs and there are contributions from combined sewer overflows in the area. The focus of this study is the last WWTP in the lower Kraichbach River with a maximum treat ment capacity of 55′000 p.e. (population equivalent). The previous five WWTPs upstream of the river treat the sewage of approximately 130′000 p.e. The studied WWTP has standard primary and secondary treatments, with nitrogen and phosphorus removal (an activated sludge system with pre anoxic and intermittent denitrification) (83% and 96.6% of efficiency, respectively), but no advanced treatments, such as ozonation, UV, microfiltration, etc. (Urban Wastewater Treatment Directive https://uwwtd.eu/Germany/uwwtps/treatment/). The river flow in this section of the river is approximately 1.22m^3s^{-1}.

2.2. Sample collection

Five sampling campaigns were performed from February to June 2019. The samples (five upstream and five downstream) were collected once a month upstream and downstream of the WWTP, to assess the influence of the effluent on the ARG and FPB. The downstream sampling point was approximately 200 m down of the WWTP effluent discharge point. Water samples were collected with pre washed and decontaminated plastic bottles. A core sampler was used to collect the first 5 cm of sediment from the river. Biofilm samples were collected

from samplers made of a PVC box containing four glass sheets of 70 × 30 cm. The glasses were previously clean to avoid any contamination. The samplers were designed and built for this purpose in our department. The glass is an artificial substrate for biofilm growth, however, the microorganisms are able to attach to any kind of material, and since environmental factors has a stronger influence on biofilm structure and composition than the attached substrate (Hempel et al., 2010), we believe our sampler are representative of the biofilms found on the ecosystem. The image of the sampler is available in the Supplementary Material, Fig. S1. The passive sampler was submerged for approximately one month while the biofilm grew on the glass sheets and was later scratched from the glass with a stainless steel spatula.

Shares of the sediment and biofilm samples were stored in previously washed and decontaminated plastic recipients and frozen at −20 °C, to preserve the DNA content of the samples. The analyzed amounts of sediment and biofilm ranged from 0.10 to 0.41 g and 0.0048 to 0.078 g (dry weight), respectively. The water samples were filtered using a vacuum pump system and polycarbonate membranes with a pore size of 0.2 μm (Whatman® Nuclepore™ Track Etched Membranes, Sigma Aldrich, Munich, Germany). The samples were filtrated until the membranes were clogged. The filtrated volume varied from 400 to 250 mL. The membranes containing the samples were frozen at −20 °C in decontaminated plastic recipients until analysis. All samples were filtered immediately after collection and arrival at the laboratory.

2.3. Physical and chemical parameters

In the water phase, pH, conductivity, temperature, and dissolved oxygen (DO) were measured in the field with a portable probe Multi 340i (WTW, Weilheim, Germany). Turbidity was measured with a portable turbidimeter (Hach, Loveland, United States). The concentration of suspended solids (SS) was measured by filtrating the samples with glass fiber filters (0.45 μm) and drying the filters at 110 °C. In the water samples, orthophosphate, total phosphorus, ammoniacal nitrogen, nitrate, and total nitrogen were analyzed with ready to use cuvette tests and a DR2800 spectrophotometer (Hach Lange, Düsseldorf, Germany). In sediment, Kjeldahl nitrogen was analyzed according to the standard DIN EN 25663 methodology, with a digestion unit (Büchi, Lawil, Switzerland). Total phosphorus was also analyzed, according to the standard methodology DEV D11 7. Both methodologies are from the *Deutsche Einheitsverfahren zur Wasser , Abwasser und Schlammuntersuchung*.

Daily values for UVA and UVB radiation (J/m^2) were obtained from *Umweltbundesamt* and *Bundesamt für Strahlenschutz* (Oberschleißheim, Germany). Mean values for the sampling periods (while the biofilm was submerged) were calculated.

2.4. Extraction of DNA and quantification of ARG

DNA from sediment, biofilm, and filtration membranes was extracted using the FastDNA™ Spin Kit for soil (MP Biomedicals, Illkirchen France) according to the kit protocol. After extraction, DNA concentration and purity were measured with Nanodrop ND 1000 Spectrophotometer (Peqlab Biotechnologie GmBH, Erlangen, Germany). The samples with low concentrations were measured with the Qubit™ 3.0 (Thermo Fisher Scientific, Nidderau, Germany). When necessary, the samples were diluted with nuclease free water (Thermo Fisher Scientific) to reach ~50 ng/μL of DNA concentration.

The quantification of ARG was performed by qPCR (quantitative polymerase chain reaction) in a Cycler CFX96 Touch™ Deep Well Real Time PCR Detection System (Bio Rad, Munich, Germany). The samples were analyzed in technical duplicates. The mixture consisted of 2 μL of the sample (or template DNA), 10 μL of Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), 1 μL of Primer FW (10 μM), 1 μL of Primer Rev. (10 μM) and 6 μL of Nuclease Free water (Thermo Fisher Scientific). For each ARG, a control (blank sample) was added to the plate.

The thermocycler conditions were: heating at 95 °C for 10 min, 95 °C for 15 s and cooling down to 60 °C for 1 min for primer annealing and elongation. The melting curve, for control of specificity, consisted of 65 °C for 5 s and heating until 95 °C (increasing 0.5 °C/s). The data analysis was performed using the Bio Rad CFX Manager Software (version 3.1). Antibiotic resistance markers were analyzed, as well as taxonomical marker genes of FPB. The analyzed parameters are listed in Tables 1 and 2.

The methodology based on Hembach et al. (2017) and Hembach et al. (2019) was used to calculate cell copies (taxonomic gene markers) and gene copies (ARGs). To calculate the number of cell or gene copies, reference strains carrying the genes and the genome sizes were used to calculate the number of cell copies. A base pair average molecular weight of 650 g/mol, and a converting factor of 10^9 ng/g were also used in the equation:

$$Cell/gene\ copies \quad \frac{amount\ of\ DNA\ [ng] * Avogardo's\ number}{average\ size\ of\ genome[bp] * 10^9 * 650}$$

Avogadro's number $= 6.022 \times 10^{23}$ molecules/mol.

The previous equation was used to create a correlation between the amount of DNA in the calibration solutions and the corresponding cell or gene copies (Supplementary Material, Table S1). The primers sequences, coefficient of determination, curves, limits of detection (LOD), and genome sizes can also be found in the Supplementary Material (Tables S1 and S2).

2.5. Antimicrobial susceptibility test

As additional information to qPCR, we performed antimicrobial susceptibility tests to detected if there were multi drug resistant bacteria in the samples. Only sediment and biofilm samples were analyzed since the water samples did not have enough material to perform both of analyses. The multi drug resistance test was performed according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) disk diffusion test (EUCAST, 2017).

Small shares of the samples were collected with disposable pre sterilized sticks and placed in falcon tubes in Muller Hinton Bouillon broth (10.5 g in 500 mL of deionized water, placed in the autoclave at 121 °C). The falcon tubes were placed in a shaker (37 °C) and left overnight. The bacteria were inoculated in CHROMagar™ ESBL (Extended Spectrum Beta Lactamase) plates (Mast Diagnostica, Reinfeld, Germany) and grew overnight at 37 °C. Of the 20 samples, four samples were selected for analysis, two biofilm samples (one upstream and one downstream of the WWTP) and two sediment samples (one upstream and one downstream of the WWTP). The species identification was performed according to CHROMagar™ ESBL Instructions for Use. The identification is based on the colony appearance (color). From each sample, 10 isolates were picked (with sterile pipette tips) and cultivated in tubes with Luria Bertany broth in a shaker overnight at 37 °C. Afterward, the colony suspension was diluted with Luria Bertany broth to

Table 1
Analyzed parameters in qPCR – genes and related facultative pathogenic bacteria.

Gene marker	Bacteria
ddl	*Enterococcus faecalis*
yccT	*Escherichia coli*
ecfX	*Pseudomonas aeruginosa*
gltA	*Klebsiella pneumoniae*
secE	*Acinobacter baumanni*
23S rRNA	Enterococci
16S rRNA	Eubacteria

Table 2
Analyzed parameters in qPCR – genes and related antibiotic resistance.

Target gene	Resistance
bla_{TEM}	Ampicillin
ermB	Erythromycin (macrolide)
tetM	Tetracyclin
sul1	Sulfamethoxazole
$bla_{CMY\ 2}$	Cephalosporin
$bla_{CTX\ M}$	Cephalosporin
$bla_{CTX\ M\ 32}$	Carbapeneme
$bla_{OXA\ 48}$	Extended-spectrum β-lactams/Carbapenems
mecA	ß-Lactams, Methicillin
$bla_{NDM\ 1}$	Highly potent extended-spectrum β-lactams/Carbapenems
$bla_{KPC\ 3}$	Extended-spectrum β-lactams/Carbapenems
mcr-1	Colistin/polymyxin

achieve an absorbance of 0.1 at 625 nm in a U 5100 Spectrophotometer (Hitachi, Tokyo, Japan). The bacteria were inoculated in Muller Hinton agar plates with sterilized cotton swabs, and antibiotic disks (Mastdiscs™ AST, Mast Group Ltd., Merseyside, United Kingdom) were applied to the agar plates and were left overnight at 37 °C.

The following 14 antibiotics were tested: Cefotaxime (5 μg), Ceftaz idime (10 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Imipenem (10 μg), Meropenem (10 μg), Piperacillin (30 μg), Piperacillin/Tazobac tam (30/6 μg), Temocillin (30 μg), Amikacin (30 μg), Tigecycline (15 μg), Trimethoprim/Sulfomethoxazole (1.25/23.75 μg), Chloram phenicol (30 μg), and Fosfomycin/Glucose 6 phosphate (200/50 μg). The inhibition zones diameters were measured and compared to the breakpoint tables, given the susceptibility of the bacteria to each antibi otic (EUCAST).

The bacteria were classified according to the Commission for Hospital Hygiene and Infection Prevention (*Kommission für Krankenhaushygiene und Infektionsprävention* KRINKO). It considers four relevant antibiotic groups and some substances: (i) acylureidopenicillins with the substance piperacillin, (ii) 3rd/4th generational cephalosporins with the substance cefotaxime and/or ceftazidime, (iii) carbapenems with the main substance imipenem and/or with meropenem, and (iv) fluoroquinolones the substance ciprofloxacin. It classifies resistant bacteria in 3MRGN (multidrug resistant Gram negative strains with resistance to three of the four antibiotic groups) and 4MRGN (multi resistant Gram negative strains resistant to all four antibiotic groups including pan resistance) (Robert Koch Institute, 2012).

2.6. Statistical analysis

Correlation analysis was performed with Statistica 10.0 software (StatSoft. Inc., Tulsa, USA). Correlations between parameters were con sidered significant with a $p < 0.05$. The boxplot graphs were built with Python libraries Matplotlib and Pandas. The rectangle represents values from the first quartile (Q1) to the third quartile (Q3) of the data. The geometric mean values are represented by the orange line inside the rectangle, the whiskers indicate the data range (minimum and maxi mum values) and circles show outliers.

3. Results and discussion

3.1. Physical and chemical parameters

According to the results, several physical and chemical parameters had similar values upstream and downstream of the WWTP (Supple mentary Material, Table S3). The concentrations of dissolved oxygen (DO) and ammoniacal nitrogen were the most affected by the effluent discharged in the river. The mean concentration for DO upstream of the WWTP was 9.28 ± 1.13 mg L^{-1}, while in the downstream samples it was 8.86 ± 1.64 mg L^{-1}. Ammoniacal nitrogen concentrations were lower upstream, with a mean concentration of 0.06 ± 0.08 mg L^{-1}, and higher downstream, with a mean concentration of 0.24 ± 0.27 mg L^{-1}. Total nitrogen and nitrate had similar values both upstream and downstream, and the values did not vary during the sam pling campaigns. The decrease in DO and the increase in nitrogen con centrations in surface water is a common effect of the discharge of WWTP effluent in rivers (Atashgahi et al., 2015; dos Santos et al., 2016).

Besides the influence of the WWTP, many parameters showed great variation throughout the sampling period, most likely due to seasonal changes. Turbidity, for example, was around 25 NTU in February and March and decreased to 2 NTU in June. The concentration of SS was also high in winter, reaching 38 mg L^{-1} in February, and decreasing to 1.23 mg L^{-1} in June. This difference is related to the decreasing river flow and the increasing abundance of macrophytes in the river starting in April. The macrophytes lower flow velocity and facilitate the sedi mentation of solid particles, and also act as a filter for SS. The DO concen tration also decreased in the warmer months, going from 9.60 mg L^{-1} in February to 6.10 mg L^{-1} in June, due to the higher temperatures. Nitrate concentrations also decrease from February (6.9 mg L^{-1}) to June (3.5 mg L^{-1}), while orthophosphate had a peak concentration in June (0.2 mg L^{-1}). The other nutrients, both in water and sediment samples, did not show important variations during the analyzed months.

3.2. Quantification of eubacterial and facultative pathogenic bacteria gene markers in water, sediment, and biofilm

The mean occurrence of the eubacterial 16S rRNA gene throughout the sampling period was $4.81 \times 10^5 \pm 4.08 \times 10^5$ cell copies/100 mL in water, $3.77 \times 10^7 \pm 5.60 \times 10^7$ cell copies/g of sediment, and $2.83 \times 10^8 \pm 3.50 \times 10^8$ cell copies/g of biofilm samples. The boxplots for the FPB quantified in the samples are depicted in Figs. 1 3. The de tailed results can be found in the Supplementary Material (Table S4).

In water samples (Fig. 1), the abundance and diversity of species in creased downstream of the WWTP. There was already a background concentration for most analyzed species in the surface water of the river, most likely because of previous wastewater emissions upstream of the investigation site. The gene marker *glt*A, of the bacterium *K. pneumoniae,* was not detected in any of the samples collected up stream of the WWTP, but it was detected with high abundances in three sampling campaigns downstream of the WWTP. For the genes de tected both upstream and downstream of the WWTP, the mean concen trations of all taxonomic marker genes were higher after the effluent discharge. In previous studies, the increase in the abundances of FPB in the environment was found due to the discharge of WWTPs, even with advanced treatments, like ozonation (Brown et al., 2019; Jäger et al., 2018).

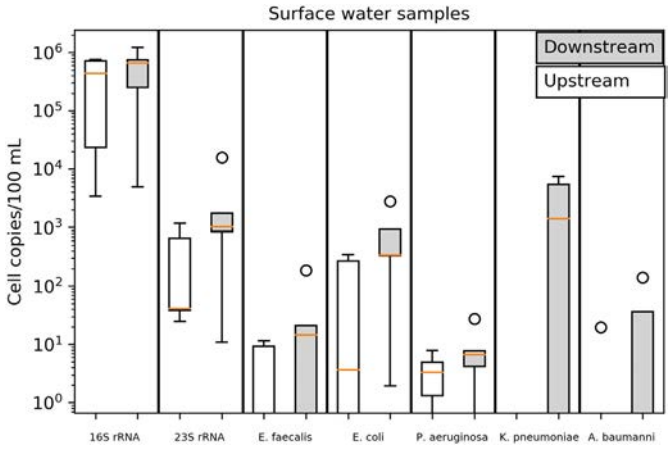

Fig. 1. Absolute abundance of taxonomical gene markers in water samples from the Kraichbach River.

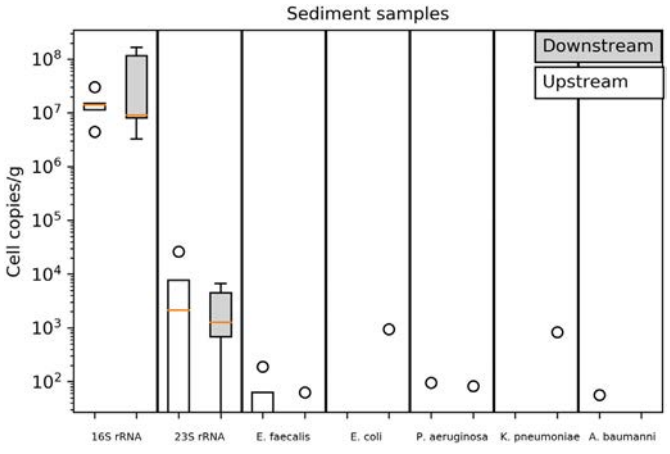

Fig. 2. Absolute abundance of taxonomical gene markers in sediment samples from the Kraichbach River (values in dry weight).

In sediment (Fig. 2), the mean values for the gene markers *23S rRNA* (*enterococci*) and *16S rRNA* were similar before and after the WWTP. Only a few FPB were detected in the samples and some gene markers were detected occasionally, mainly in February and March (Table S4). The gene markers for *E. coli* and *K. pneumoniae* were detected only after the discharge of the effluent, but it is not possible to see a strong influence of the WWTP in the FPB detected in sediment samples. Usually, WWTPs with higher treatment capacities have a stronger influence on the presence of FPB in environmental samples. For example, in a similar research in Germany, the abundances of three gene markers in sediment samples were strongly affected by the effluent discharge, however, the size of the WWTP was much bigger, with a treatment capacity of approximately 875′000 p.e. (Brown et al., 2019).

Similar to sediment, biofilms can also act as reservoirs and sources of important pathogenic species, and aquatic biofilms were already identified as important niches for pathogenic bacteria (Abraham, 2011). In biofilm samples (Fig. 3) the detection frequency and abundances of FPB were higher than in sediment. Although the mean values of *23S rRNA* and 16*S rRNA* were similar before and after the discharge of the WWTP, the occurrence of FPB was higher in samples collected downstream of the WWTP. The only FPB that was not detected above the limit of detection (LOD) in any biofilm sample was *K. pneumoniae* (*glt*A). *E. faecalis* (*ddl*) was detected in three samples upstream of the WWTP, and *E. coli* (*ycc*T) was detected downstream of the WWTP only.

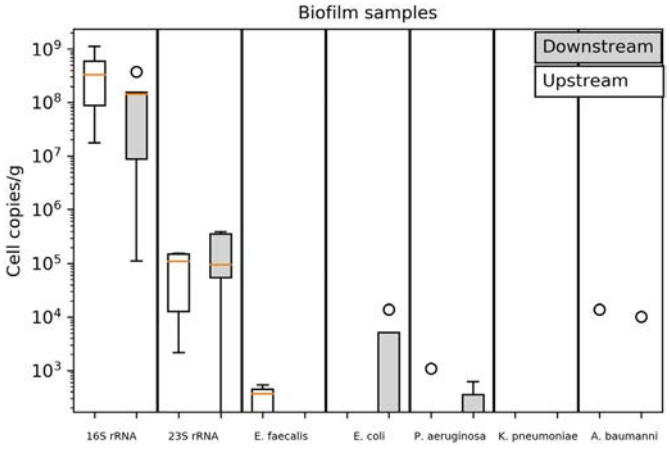

Fig. 3. Absolute abundance of taxonomical gene markers in biofilm samples from the Kraichbach River (values in dry weight).

3.3. Quantification of ARG in water, sediment, and biofilm

The boxplots for the ARG quantified in the samples are depicted in Fig. 4 6. The detailed results can be found in the Supplementary Material (Table S5).

Because of the intense use of some antibiotics, the resistance genes linked to them are usually detected more frequently (Hembach et al., 2019). This is the case with the bla_{TEM}, *ermB, tet*M, and *sul*1 genes, encoding resistance to β lactams, erythromycin, tetracycline, and sulfamethoxazole (Hembach et al., 2019). These ARGs were detected in all water, sediment, and biofilm samples analyzed (Fig. 4 6).

According to the results, the abundance of ARGs was higher in water samples collected downstream of the WWTP than in those collected upstream of the WWTP (Fig. 4). The highest concentrations detected in water samples were of *erm*B and *sul*1, with mean concentrations of 3.94×10^5 and 2.50×10^5 gene copies/100 mL in samples collected downstream of the effluent discharge. In previous works, *erm*B was found to be very abundant in a German river, also reaching approximately 10^6 gene copies/100 mL in surface water samples (Brown et al., 2019). However, *erm*B is not always detected in other countries, but the gene *sul*1 was frequently detected in China. In the Yangtze Estuary, *sul*1 was detected with a concentration of up to 3.19×10^7 gene copies/100 mL (Guo et al., 2018) and in northeastern China, the concentrations of *sul*1 reached 3×10^5 gene copies/100 mL (Lu et al., 2015).

The other ARGs were usually only detected in water samples collected downstream of the WWTP, while most of the concentrations detected upstream were below the LOD. Of these less frequent genes, bla_{OXA-48} was detected with higher concentrations, of up to 1.29×10^4 gene copies/100 mL in the Kraichbach River. In a recent study in Europe, bla_{OXA-48} was detected in surface waters with an approximate median of 5×10^4 gene copies/mL and was the 6th most abundant gene after genes like *sul*1, *tet*M, and bla_{TEM}, (Cacace et al., 2019). A very important ARG, *mcr 1,* was detected in two samples collected downstream of the effluent discharge. This gene was discovered recently in China and is linked to the resistance against a last resort antibiotic. It was already detected in WWTP effluents in Europe (Cacace et al., 2019; Hembach et al., 2017; Lekunberri et al., 2017) but is hardly detected in environmental samples (Yang et al., 2017).

In sediment samples, only the most frequent ARGs were detected above the LOD (Fig. 5). The mean concentrations detected in samples collected upstream and downstream of the WWTP were very similar and the values did not have a great variation during the sampling period. Like in water samples, the highest concentrations were of *erm*B and *sul*1. The mean concentrations for *erm*B and *sul*1 were 4.82×10^6 and 2.69×10^6 gene copies/g for both locations, while mean concentrations of *tet*M, and bla_{TEM}, were approximately 10^4 gene copies/g. In another river in Germany, impacted by a bigger WWTP, the concentrations of *tet*M, and bla_{TEM} were also similar, around 10^5 gene copies/g upstream and 10^6 gene copies/g downstream of the WWTP (Brown et al., 2019).

These patterns for *sul*1 and other ARGs are comparable with those found in recent studies (Cacace et al., 2019; Marti et al., 2013). The authors believe urban rivers to have background contamination and to be filled already with ARGs related to commonly prescribed antibiotics. In this case, the discharge of effluents would not significantly influence the occurrence of ARGs, mainly in sediment, which slowly responds to changes in the environment. Since the Kraichbach River has other effluent sources upstream of the studied section, as well as agricultural areas, these ARGs may be abundant in the whole basin, as the high detection frequencies and concentrations upstream of the WWTP suggest.

The ARGs *erm*B, *sul*1, *tet*M, bla_{TEM}, bla_{OXA-48}, and bla_{KPC-3} were detected in biofilm samples (Fig. 6). Many of the other ARGs were detected in the samples, but the values were below the LOD. The abundances of the main four ARGs were at least one order of magnitude higher in biofilm than in sediment samples. The ARG bla_{KPC-3} was detected in biofilm samples, but not on water or sediment samples. This can happen because inside aquatic ecosystems, bacteria may stay in their free life

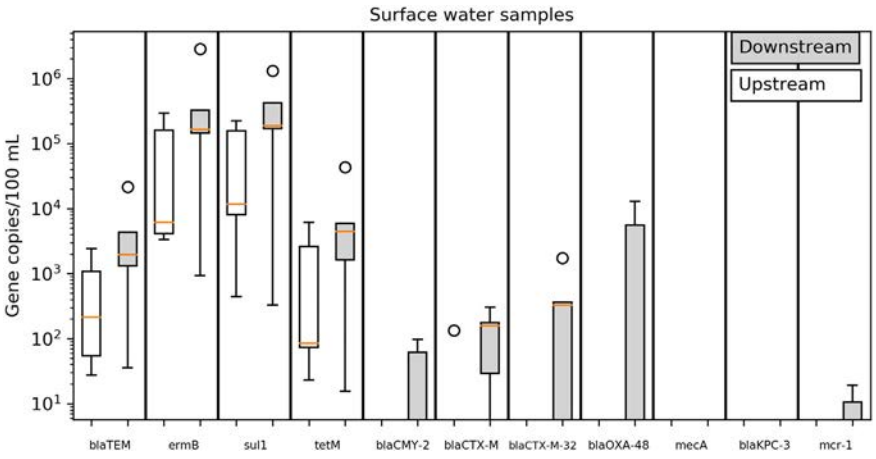

Fig. 4. Absolute abundance of ARGs in water samples from the Kraichbach River.

forms or attach to surfaces, forming biofilms. The bacteria present in the biofilm may also detach later, depending on the maturation stage of the biofilm, food and oxygen availability, etc. (Toyofuku et al., 2016). The results emphasize the importance of analyzing different media when looking for FPB and pollutants in an ecosystem.

Some of these ARG were already analyzed in biofilm Guo et al. (2018), who also found higher concentrations of *sul*1 and other ARGs in biofilm than in sediment samples. The abundances of bla_{OXA-48}, and bla_{KPC-3} were lower, reaching 5.67×10^4 gene copies/g of bla_{OXA-48} and 6.61×10^3 gene copies/g of bla_{KPC-3}. These two genes were also analyzed in biofilm samples from two rivers in Spain, impacted by raw and treated sewage. The concentrations of bla_{KPC-3} were approximately 10^5 gene copies/g, but bla_{OXA-48} was not detected in any sample (Subirats et al., 2017a). Although the occurrence and abundance of ARGs may be different in biofilm samples from different locations, the results show that biofilms can be important disseminators of antibiotic resistance in urban rivers and may play an important role in the spread of these genes in the environment because of their dispersion mechanisms and adaptability to other ecosystems.

3.4. Antimicrobial susceptibility test multi drug resistance

This was a qualitative test since not all of the samples and isolates were analyzed. Two sediment samples and two biofilm samples (one upstream and one downstream) were chosen to perform the antimicrobial susceptibility tests with cultivable isolates previously enriched from population communities. However, not all of the isolates presented bacterial growth in the last stage of the analysis (antibiotic application), probably due to the low temperature the samples were exposed for preservation. The detailed results from the antimicrobial susceptibility test, including inhibition zones and MRGN results, are displayed in the Supplementary Material (Tables S6 S8).

The isolates chosen in the sediment sample collected upstream of the WWTP were classified as KEC *Klebsiella, Enterobacter, Citrobacter* (three isolates), *E. coli* (three isolates), and *Acinetobacter* (three isolates, one did not present any bacterial growth). Many isolates were resistant to cephalosporins (either cefotaxime or ceftazidime, or both). No isolate collected upstream of the WWTP was classified as 3MRGN or 4MRGN. Downstream of the WWTP, the isolates belonged to the species *P. aeruginosa* (five isolates) and *Acinetobacter* (five isolates, three did not present any bacterial growth). Two *P. aeruginosa* isolates were classified as 3MRGN, one was resistant to cephalosporins, meropenem and piperacillin and the other was resistant to cephalosporins, ciprofloxacin, and meropenem.

E. coli was the only species successfully isolated from the biofilm sample collected upstream of the WWTP and only three isolates had bacterial growth. These three isolates were resistant to cefotaxime, ceftazidime, and fosfomycin. Downstream of the WWTP, *Acinetobacter* (three isolates), and KEC (six isolates) were analyzed. One of the KEC isolates in the biofilm sample collected downstream of the WWTP was classified as 3MRGN and was resistant to cefotaxime, ciprofloxacin, and piperacillin.

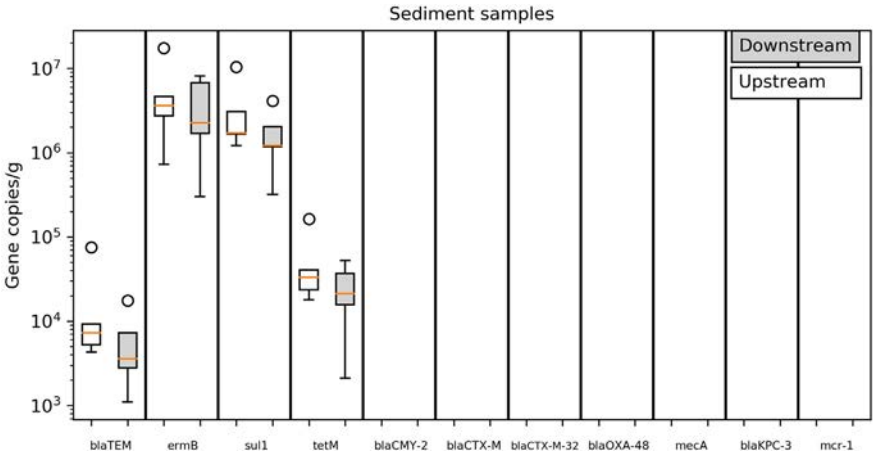

Fig. 5. Absolute abundance of ARGs in sediment samples from the Kraichbach River.

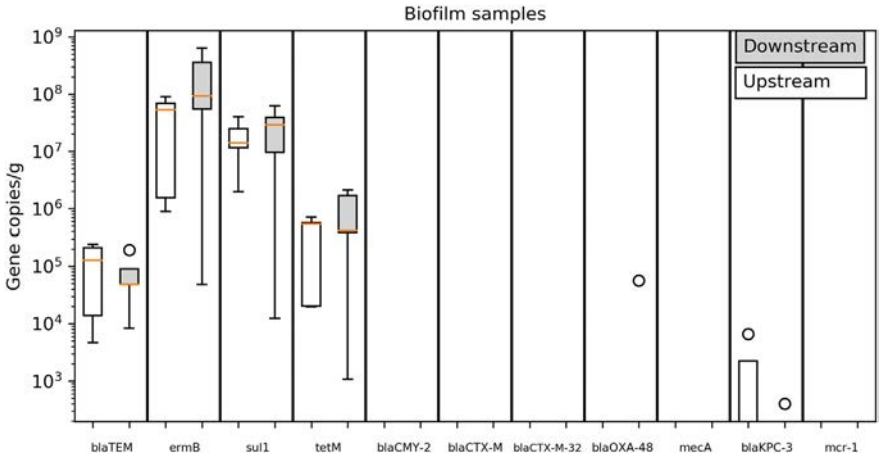

Fig. 6. Absolute abundance of ARGs in biofilm samples from the Kraichbach River.

There was a clear impact of the WWTP on the dissemination of multi resistant bacteria into the aquatic environment. 3MRGN isolates were only detected downstream of the effluent discharge both in sediment and biofilm. The presence of multi resistant bacteria is a potential health risk in case of human infection. Since biofilms can survive in almost every ecosystem and the microorganisms found in biofilms may be mobile, the ARGs could overpass the natural/anthropogenic frontiers. Therefore, the presence of antibiotic resistance and multidrug resistant bacteria in stream biofilm can represent a threat to human and animal health.

3.5. Correlations of ARGs and FPB with physical and chemical parameters

The complete correlation table can be found in the Supplementary Material. The antimicrobial susceptibility test was not included in the statistical analysis because it was not quantitative and only four samples were analyzed. We performed the correlation analysis separately for the samples collected upstream and downstream of the WWTP since the results seemed to be different.

Many parameters in the studied river had a great variation along the year, such as turbidity, SS, water temperature, radiation, the presence and quantity of macrophytes, etc. During the winter, turbidity and SS reach their peaks in the Kraichbach River. We found that turbidity was strongly correlated ($r > 0.906$, $p < 0.034$) with several ARGs and FPB detected in water samples collected upstream of the effluent discharge (except for bla_{CTX-M}, 16S rRNA and *sec*E). In these samples, it can be noticed that the diversity and abundance of ARGs and FPB were higher at the beginning of the year (February, March), but there is a decreasing tendency until June (Supplementary Material, Tables S4 S5). However, these correlations were not detected for the water samples collected downstream of the WWTP.

The concentration of SS also correlates with the ARGs *erm*B, *sul*1, *tet*M, $bla_{TEM,}$ ($r > 0.883$, $p < 0.047$) and the gene markers 23S rRNA and yccT ($r > 0.880$, $p < 0.049$) detected in water samples upstream of the WWTP. Downstream of the WWTP, no correlation with SS in water samples was found, but significant correlations were detected for *erm*B, 16S rRNA and 23S rRNA collected from sediment ($r > 0.887$, $p < 0.045$). These positive correlations indicate that turbidity and SS are possible causes of higher ARGs and FPB abundances during winter before the effluent discharge, but not after. A significant part of the riverine microbial community attaches to suspended solids, because of the organic matter fraction, a source of food for these organisms (Peduzzi and Luef, 2008). Also, higher turbidity and SS values prevent sunlight and radiation from penetrating the water column.

Significant negative correlations ($p < 0.05$) between UVA radiation, ARGs and FPB (23S rRNA, *ddl*, *ycc*T, *erm*B, and *sul*1) in upstream water samples were also detected. These correlations do not apply for samples collected downstream of the WWTP. UV radiation is known to decrease freshwater bacterial growth and production (Hörtnagl et al., 2011) and as the temperature (and UV radiation) gets higher closer to spring and summer, macrophytes grow on the bottom of the river, filtrating the water and decreasing the river flow. This can also help in the self cleaning capacity of the river, which may act like a sedimentation basin, reducing the concentrations of suspended solids in water (Franklin et al., 2008).

Regarding nutrients, only ammoniacal nitrogen seems to influence the results. It correlated with some ARGs and FPBs in water samples (23S rRNA, *ddl*, *ycc*T, *erm*B, and *sul*1, $r > 0.892$ $p > 0.041$) and biofilm samples (bla_{TEM} and *sul*1, $r > 0.934$ $p > 0.020$) collected upstream. Downstream of the WWTP, the influence was stronger and ammoniacal nitrogen had significant correlations with all the ARGs and FPB, except for 16S rRNA and bla_{CMY-2}. Since ammoniacal nitrogen is an wastewater indicator in water resources (Ide et al., 2017), and the correlations were stronger after the effluent discharge, the results suggest that the detection of ARGs and FPB are connected to effluent or sewage discharges into the river, mainly downstream of the WWTP. Higher bacterial and ARG diversity were also detected downstream of a WWTP in Spain (Marti et al., 2013), supporting the idea that the nutrients, bacteria, and ARGs released by the WWTP have an important influence on the microbial community of streams and rivers (Brown et al., 2019; Cacace et al., 2019; Lapara et al., 2011).

Significant correlations were observed more frequently in ARGs and FPB detected in water samples because the occurrence of the target genes was higher in these media. Also, the occurrence of ARGs and FPB in sediment was at the same level during the sampling campaigns, indicating that the microbiota in these media are not influenced by external parameters. These results indicate that the occurrence of ARGs and FPB upstream and downstream of the WWTP, especially in surface water, is influenced by the water quality, but in different ways. Parameters that changed according to seasons, like turbidity, SS, and UVA had stronger correlations with ARGs and FPB detected in samples collected upstream of the effluent discharge. In these samples, higher abundances of ARGs and FPB were detected during the winter, when turbidity and SS values were also high, and the UVA radiation was low. This indicates that high turbidity and concentration of suspended solids, along with low sunlight and UVA radiation, can favor the presence and the spread of FPB and ARGs in surface water. Downstream of the WWTP, ammoniacal nitrogen, a parameter linked to the water quality and the presence of domestic effluents, had higher correlations with the abundance of ARGs and FPB. This indicates that in this section of the river, seasonal parameters do not have a major impact on the microbiota, and that the input of effluent by the WWTP has a strong influence on the presence of ARGs and FPB.

4. Conclusion

Our results showed that the presence and abundance of ARGs and FPB in the Kraichbach River were influenced by the water quality and seasonal parameters. The ARGs and FPB detected in water samples up stream of the WWTP were more susceptible to changes in the environ ment, and had higher correlations with parameters that showed seasonal variations, such as turbidity, suspended solids, and UVA radia tion. Downstream of the WWTP, the concentration of ammoniacal ni trogen, and consequently the presence of effluent in the river, seemed to play a major role on the occurrence of ARGs and FPB. The genes de tected in biofilm and sediment samples presented few significant corre lations with water quality or seasonal parameters, indicating water grab samples can be used to detect temporal changes, contamination points and sensitive areas.

The occurrence of ARGs in sediment was low and did not vary much during the sampling campaigns. One recommendation to improve and maximize the use of sediment as monitoring media for ARGs and FPBs is to collect only the upper layer of sediment (less than 1 cm), where bacteria usually are more abundant. Biofilm had higher abundances of the target genes when compared to sediment, which indicates biofilm can act as a sink of ARGs and FPB in aquatic ecosystems. The occurrence of the target genes in sediment and biofilm samples was similar before and after the effluent discharge. However, multi drug resistance was mainly detected in the isolates collected from the biofilm and sediment samples collected downstream of the WWTP, reinforcing the idea that WWTPs are hot spots for antibiotic resistance. Also, biofilm samples can be a more accurate representation of the presence of ARGs and FPB in the basin, since it is intrinsically connected to the microbiota in the river.

The biofilm sampler was found to be a cheap and useful tool that al lows collecting biofilm in rivers, streams, and even in artificial channels and waterways, where there are no cobbles or other submerged struc tures to collect biofilm from. It also acted as a passive sampler and helped to collect biofilm from pre determined time frames and seasons along the year, integrating time, location, and water flow, giving a more complex response than water and sediment sampling. However, when planning biofilm monitoring campaigns in other areas, differences in temperature, sunlight, and nutrient availability should always be con sidered to determine the adequate minimum exposure time for biofilm to grow inside a river.

CRediT authorship contribution statement

Gabriela Reichert: Conceptualization, Methodology, Formal analy sis, Investigation, Data curation, Writing review & editing. **Stephan Hilgert:** Conceptualization, Methodology, Investigation, Writing review & editing. **Johannes Alexander:** Conceptualization, Methodol ogy, Validation, Writing review & editing. **Júlio César Rodrigues de Azevedo:** Funding acquisition, Supervision. **Tobias Morck:** Funding acquisition, Writing review & editing. **Stephan Fuchs:** Conceptualiza tion, Resources, Funding acquisition, Writing review & editing, Supervision. **Thomas Schwartz:** Methodology, Validation, Resources, Writing review & editing.

Declaration of competing interest

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

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