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Hospital and urban wastewaters shape the matrix and active resistome of environmental biofilms

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ABSTRACT

Understanding the dynamics of antibiotic resistance gene (ARG) transfer and dissemination in natural environments remains challenging. Biofilms play a crucial role in bacterial survival and antimicrobial resistance (AMR) dissemination in natural environments, particularly in aquatic systems. This study focused on hospital and urban wastewater (WW) biofilms to investigate the potential for ARG dissemination through mobile genetic elements (MGEs). The analysis included assessing the biofilm extracellular polymeric substances (EPS), microbiota composition as well as metatranscriptomic profiling of the resistome and mobilome. We produced both *in vitro* and *in situ* biofilms and performed phenotypic and genomic analyses. In the *in vitro* setup, untreated urban and hospital WW was used to establish biofilm reactors, with ciprofloxacin added as a selective agent at minimal selective concentration. In the *in situ* setup, biofilms were developed directly in hospital and urban WW pipes.

We first showed that a) the composition of EPS differed depending on the growth environment (*in situ* and *in vitro*) and the sampling origin (hospital vs urban WW) and that b) ciprofloxacin impacted the composition of the EPS. The metatranscriptomic approach showed that a) expression of several ARGs and MGEs increased upon adding ciprofloxacin for biofilms from hospital WW only and b) that the abundance and type of plasmids that carried individual or multiple ARGs varied depending on the WW origins of the biofilms. When the same plasmids were present in both, urban and hospital WW biofilms, they carried different ARGs. We showed that hospital and urban wastewaters shaped the structure and active resistome of environmental biofilms, and we confirmed that hospital WW is an important hot spot for the dissemination and selection of antimicrobial resistance. Our study provides a comprehensive assessment of WW biofilms as crucial hotspots for ARG transfer. Hospital WW biofilms exhibited distinct characteristics, including higher eDNA abundance and expression levels of ARGs and MGEs, highlighting their role in antimicrobial resistance dissemination. These findings emphasize the importance of understanding the structural, ecological, functional, and genetic organization of biofilms in anthropized environments and their contribution to antibiotic resistance dynamics.

1. Introduction

Biofilms are the preferred lifestyle of bacteria in the natural

environment. They present structures that support high densities of bacteria, exhibit high tolerance to antimicrobials, and facilitate gene transfer (Balcazar et al., 2015; Hennequin et al., 2012). In aquatic

Abbreviations: Untreated wastewater, WW; antimicrobial resistance genes, ARGs; extracellular polymeric substances, EPS; mobile genetic elements, MGEs; WW treatment plants, WWTP; extracellular DNA, eDNA; horizontal gene transfer, HGT; antimicrobial resistance, AMR; minimal selective concentration, MSC.

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environments, biofilms are suggested to serve as hot spots for the accumulation and dissemination of antimicrobial resistance genes (ARG) (Abe et al., 2020; Balcazar et al., 2015). Chemical pollutants spread by urban, industrial, and agricultural waste exert a continuous selective pressure on the diverse ARG pool present in the natural environment and its microbial communities (Allen et al., 2010; Flandroy et al., 2018). For example, ciprofloxacin is a very stable molecule often found in anthropogenically polluted environments and enriched in wastewater (WW), particularly hospital WW (Buelow et al., 2020; Hayes et al., 2022). Ciprofloxacin is not efficiently removed by WW treatment through activated sludge (Kim et al., 2020); it can be detected in WW and downstream aquatic environments and was identified to pose a significant risk for selection of antimicrobial resistance (AMR) in these environments (Hayes et al., 2022; Jia et al., 2012). Furthermore, ciprofloxacin is known to induce the SOS-response in Gram-negative bacteria. This induction of the SOS response can lead to the expression of antibiotic resistance genes as the quinolone resistance-encoding *qnrB* gene, or the expression of genetic elements as the class 1 integron integrase gene, leading to ARG cassette rearrangements (Guerin et al., 2009). In addition, bacteria that are present in the human gut, carry a wide variety of ARGs that can be spread into human impacted environments by human feces through WW (Karkman et al., 2019). For example, via the irrigation with WW effluents and/or the application of biosolids and manure to crops as well as direct fecal pollution discharges from agriculture and aquaculture, antibiotic resistant bacteria (ARB), ARGs and pathogens can be directly transmitted to the aquatic and terrestrial environments. To limit the dissemination of chemical pollutants, pathogens and ARGs into the environment, the implementation of WW treatment systems worldwide plays a key role (Flandroy et al., 2018; Singer et al., 2016). However, WW treatment plants (WWTP) are also regarded as selection and dissemination hot spots due to the high density of human gut bacteria, environmental bacteria, and chemical pollutants (Haenni et al., 2022).

Biofilms dominate most habitats on earth, accounting for ~ 80% of all bacterial and archaeal cells (Flemming and Wuertz, 2019). Biofilms that form in WW pipes along the sewerage system and in WWTP represent an important source of ARGs and ARB, and present an important model to study the dissemination of resistance in the context of discharged effluents (Abe et al., 2020; Luo et al., 2021). Biofilm communities are typified by high densities of bacteria embedded in a self-produced protective matrix, which contributes to high antibiotic tolerance, and facilitates gene transfer (Ratajczak et al., 2021). Extracellular polymeric substances (EPS), such as extracellular DNA (eDNA), proteins, lipids, and polysaccharides, are essential structural components of the biofilm matrix that are involved in resilience to antibiotics and exogenous stress, but also in horizontal gene transfer (HGT) (Flemming and Wingender, 2010; Soares et al., 2019). Previous studies have shown that hospital WW contain significantly higher amounts of resistant bacteria, ARGs and mobile genetic elements (MGEs), as well as chemical and pharmaceutical pollutants compared to urban WW. However, these quantities are generally diluted when hospital WW is mixed with urban WW in community sewer systems (Buelow et al., 2020, 2018).

Demonstration of the transfer, dynamics, and regulation of ARGs and MGEs in a complex environmental matrix is yet experimentally challenging, with many essential open questions such as how and when transfer and dissemination of ARGs occur in nature. The extent and conditions of MGE transfer that carry ARGs is still largely unexplored in natural environments and complex microbial communities. We produced both *in vitro* and *in situ* biofilms and performed phenotypic and genomic analyses. In the *in vitro* setup, untreated urban and hospital WW was used to establish biofilm reactors, with ciprofloxacin added as a selective agent at minimal selective concentration (MSC). In the *in situ* setup, biofilms were developed directly in hospital and urban WW pipes for 7 days.

To better understand the role of hospital and urban WW biofilms in

disseminating AMR through WW, we explored the EPS within the biofilm matrix, the composition of the biofilm community (microbiota), the resistome (antibiotic, biocide, and heavy metal resistance genes) and mobilome (MGEs), with focus on expressed genes (metatranscriptome).

2. Materials and methods

2.1. Sample collection and biofilm production *in situ* and *in vitro*

WW biofilm production campaigns (*in vitro* and *in situ*) were conducted in January 2017. In the *in vitro* setup, untreated urban and hospital WW was used to establish biofilm reactors and in the *in situ* setup, biofilms were developed directly in hospital and urban WW pipes for 7 days (Y Fig. 1). For *in vitro* biofilms, 20 L of untreated urban (up flow of the WWTP Limoges city), and hospital (900-bed, Limoges teaching hospital, France) WW were collected on the same day and transported (at 4 °C) to the RESINFIT laboratory in Limoges for immediate use in continuous biofilm reactors. The remaining WW was stored at 4 °C to replace evaporated volume every two days. For each WW type, two reactors containing 3 L of WW were fed at a continuous rate, from an extracellular container containing the same WW (5 L in total), ensuring circulation and a stable level in the biofilm reactors (closed continuous system) (Supplementary Figure 1). Rooms were kept darkened, and the temperature was maintained at 17 °C. Sixteen polystyrene slides suitable for confocal laser scanning microscopy (CLSM) (Agar Scientific LTD, UK) were attached to a rotator that was constantly turning, producing 16 biological replicate biofilms per reactor. After 3 days of initial biofilm formation, 1 µg/L of ciprofloxacin (considered as the minimum selective concentration (MSC) of ciprofloxacin for most environmental bacteria (Bengtsson-Palme and Larsson, 2016) was added to one reactor for each biofilm type (urban, hospital). This was repeated daily, until biofilm harvest at day 7. On day 7, the urban and hospital biofilms grown in the reactors containing ciprofloxacin were challenged with a fresh dose of ciprofloxacin (1 µg/L) and harvested the same day (D7) 4 h after ciprofloxacin challenge. Samples were processed within 30 min after harvesting. For genomic analysis based on DNA and RNA (DNA based high-throughput qPCR for resistome analysis, 16S rDNA sequencing for microbiota profiling and RNA sequencing for metatranscriptomic analysis), 8 biological replica biofilms were scraped with tissue cell scrapers (Biologix®) from the slides and washed/suspended in 1X phosphate buffered saline solution (PBS), transferred in 2 ml Eppendorf tubes, and immediately shock frozen in liquid nitrogen. Samples were stored at -80 °C until RNA/DNA extraction. For CLSM analysis, the remaining 8 slides were washed gently once with 1x PBS and either directly stained (for life/dead staining) or fixed in 3.7% formaldehyde solution for 1 hour and preserved for subsequent EPS staining.

For *in situ* biofilms, holes were drilled into polystyrene slides ends and attached with fishing line (16 slides in total, Supplementary Figure 1). The string of polystyrene slides was immersed into the hospital WW pipe and attached at the access point. Slides were recovered after 7 days. Slides were separated and carefully collected in a slide box containing cooled 1xPBS solution. Slides that showed sufficient biofilm formation (by eye) and were least polluted with residues of tissue and other large waste debris, were immediately scraped from the slides and shock frozen in liquid nitrogen on site. The remaining slides were transported to the laboratory immediately for further processing. Biofilms were washed gently in 1xPBS and fixed in 3.7% formaldehyde solution for 1 hour for subsequent EPS staining and CLSM analysis. The same procedure was applied for the *in situ* biofilm production in the untreated urban WW directly at the WWTP (slides were attached and emerged in the large WW receiving pipe/body after primary water filtration step (removal of large debris)). Slides were recovered as described above and stored in a slide box containing cooled 1xPBS. At least four suitable biofilm slides/replicates were directly processed on site by scraping them from the slides and shock freezing them in a portable liquid nitrogen tank. The remaining slides were transported at

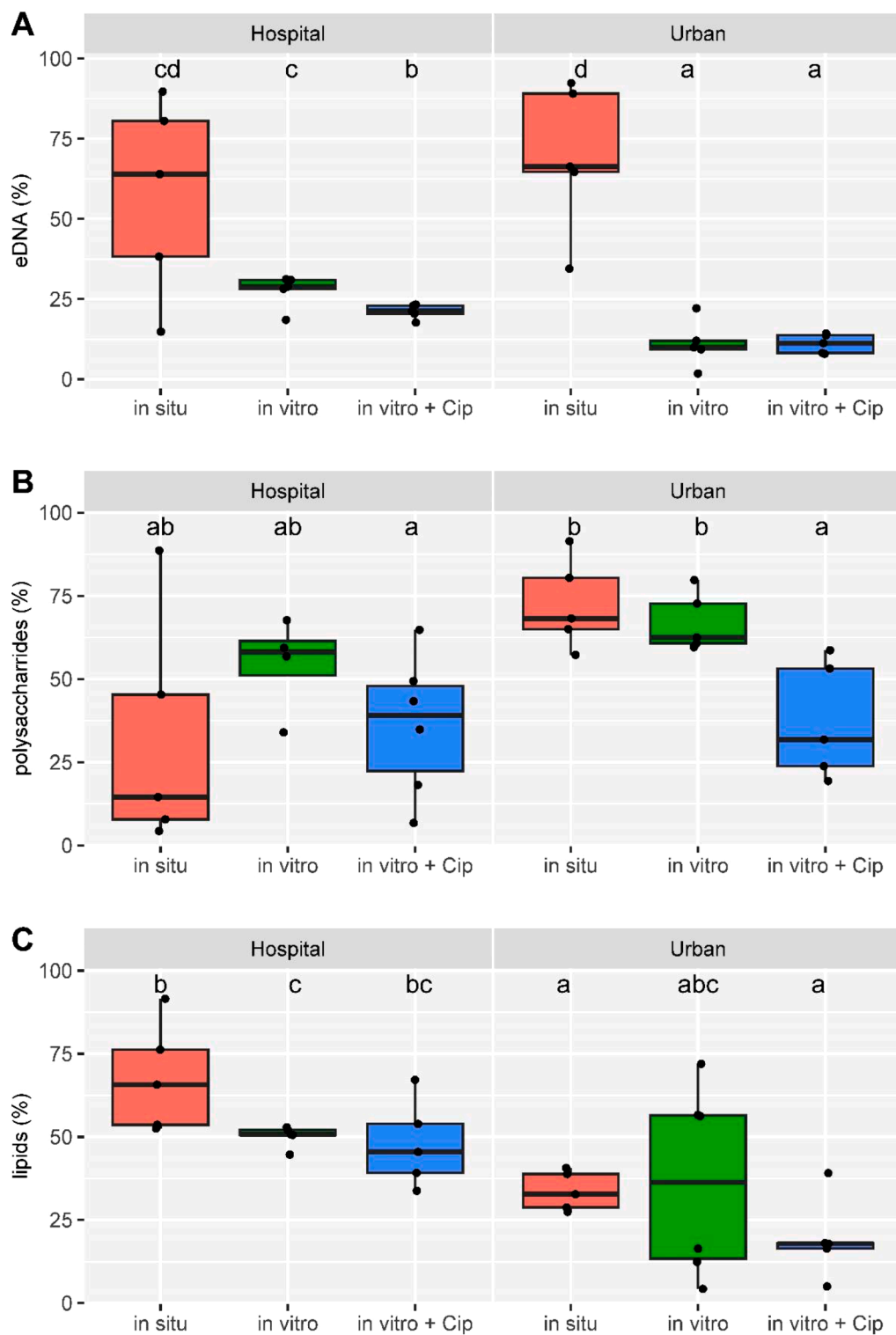


Fig. 1. Relative abundance of eDNA (A), polysaccharides (B) and lipids (C) in the respective biofilms. Each EPS component was stained in a different biological replicate, and fluorescence was detected by 5 z-stack acquisitions for each biofilm sample with a 100-fold magnification for the respective biofilms. Quantitative image analysis was performed with Biofilm Q. Results of pairwise permutations tests are summarized using the compact letter display. Samples that do not share a letter in common are significantly different from each other. +Cip= *in vitro* biofilms were exposed after 3 days of initial biofilm formation with 1 $\mu\text{g}/\text{ml}$ ciprofloxacin for 4 consecutive days and harvested after exposure of 4 h on the final day.

4 °C to the laboratory and fixed in 3.7% formaldehyde solution for subsequent EPS staining and CLSM analysis as described in the full methods section in the supplementary material.

2.2 – 2.10 The full methods concerning CLSM analysis, 16S rRNA gene sequencing, high-throughput qPCR for resistome analysis, RNA sequencing and metatranscriptomic analysis and statistical analysis are provided in the [supplementary data](#).

2.2 Extracellular polymeric substances (EPS) staining for confocal scanning laser microscopy (CSLM)

2.3 Confocal scanning laser microscopy settings and analysis

2.4 DNA/RNA extraction

2.5 16S rRNA gene sequencing and sequence data pre-processing

2.6. 16S rRNA data analysis

2.7. High-throughput qPCR for the characterization of the resistome composition and normalized abundance

2.8. Metatranscriptomics and RNA sequencing

- 2.9. Metatranscriptomic analysis
- 2.10. Statistical analysis
 - 2.10.1 Biofilm matrices
 - 2.10.2 Pairwise comparison of the normalized abundance of the resistome data using the Tukey HSD test
 - 2.10.3 Correlation of the normalized abundance for ARGs detected by qPCR and their likelihood to be expressed and detected by our metatranscriptomic pipeline
 - 2.10.4 Pairwise comparison of biofilm communities, mobilome and resistome
 - 2.10.5 Multivariate analysis for ARG detected on plasmids (mobilome)

3. Results

3.1. Effects of minimal selective concentrations of ciprofloxacin, sample origin and environment, on hospital and urban WW biofilms' extracellular polymeric substances

We first analyzed the global structure of the hospital and urban WW biofilm matrix. The EPS (eDNA, lipids and polysaccharides) were visualized by CSLM. 3-D imaging (IMARIS) and quantitative analysis (Biofilm Q) showed that the EPS significantly differ between their growth environment, namely whether biofilms were grown *in situ* or, as reconstituted biofilms, *in vitro*. We detected different relative abundance of eDNA (in both hospital and urban biofilms *in situ* compared to their *in vitro* counterparts), polysaccharides (for urban *in situ* vs *in vitro* + cip biofilms) and lipids (for hospital *in situ* vs *in vitro* biofilms) (Figs. 1 and 2, Supplementary Figures 2a and 2b, Supplementary Table 1). The relative abundance of eDNA was higher for the hospital *in vitro* biofilms compared to the urban *in vitro* WW biofilms, as well as the relative abundance of lipids for the hospital *in vitro* vs urban *in vitro* biofilms exposed to ciprofloxacin (Figs. 1 and 2, Supplementary Figure 2a). We also assessed the impact of adding ciprofloxacin on the life-dead ratio and EPS components of the *in vitro* reconstituted hospital and urban WW biofilms. A significant increase of dead cells was detected for the hospital *in vitro* biofilm after adding ciprofloxacin, compared to its control (Supplementary Figure 3). The relative abundance of eDNA was significantly lower in the hospital *in vitro* biofilm exposed to ciprofloxacin and

significantly lower for polysaccharides in the urban *in vitro* biofilm exposed to ciprofloxacin compared to their *in vitro* biofilm control.

Finally, we implemented statistical models based on random permutations testing for an overall effect of the environment (*in situ* vs *in vitro*), the sample origin (hospital vs urban) and the effect of ciprofloxacin, on the EPS components of these biofilms (Supplementary Table 1). We confirmed that i) the sample origin (hospital vs urban WW) has a significant impact on the abundance of polysaccharides, lipids, and eDNA ii) whether biofilms are grown *in situ* or *in vitro* has a significant impact on the relative abundance of eDNA and iii) ciprofloxacin has a significant impact on the abundance of polysaccharides for both, hospital, and urban *in vitro* biofilms (Supplementary Table 1).

3.2. The microbiota of hospital and urban WW *in situ* and *in vitro* biofilms

The microbial compositions of WW biofilms were assessed using 16S rDNA sequencing. In line with the phenotypic analysis of the biofilm structures, bacterial community profiles of hospital and urban *in situ* biofilms were significantly different from their *in vitro* counterparts (Supplementary Figure 4a and Supplementary Table 2). The main genera in the *in situ* hospital WW biofilms are pathogen-associated Gram-negative taxa such as *Pseudomonas*, *Acinetobacter* and *Aeromonas*, responsible for infections in hospitalized patients (Manchanda et al., 2010; Toleman et al., 2004; Vandewalle et al., 2012) (Fig. 3). *In situ* urban WW biofilms are dominated by different genera than hospital WW biofilms, namely *Flavobacterium* and *Arcobacter* but share *Acinetobacter* with hospital *in situ* biofilms. The genus *Arcobacter* is a common member of human WW systems and can rarely act as animal and human pathogen (Ferreira et al., 2013; Fisher et al., 2014). It is more abundant in urban WW biofilms, compared to the hospital WW environment. *Pseudomonas* is a dominant genus in both urban and hospital *in vitro* biofilms. Surprisingly, *Acinetobacter* decreased in the *in vitro* biofilms compared to their *in situ* counterparts. All biofilms studied, regardless of their sampling origin or environment, had a similar diversity index based on the order level (Supplementary Figure 4 b, Simpson's diversity). On genus level, which provides a higher resolution of the diversity of the microbiota, the hospital *in situ* biofilm had the lowest

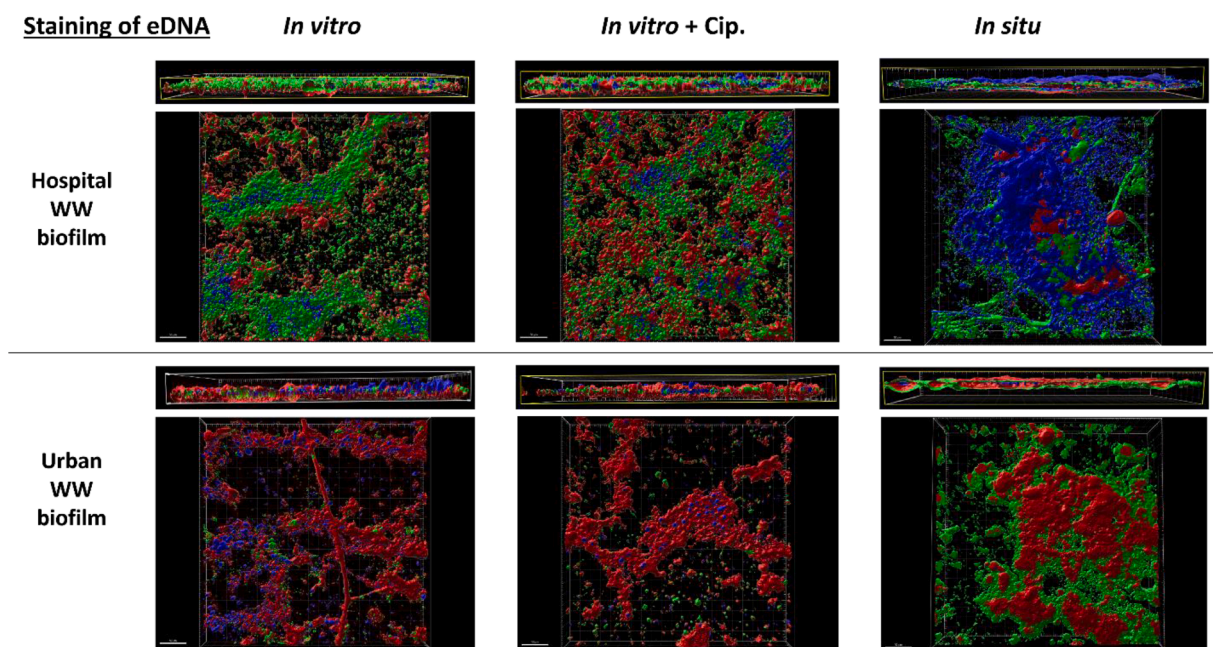


Fig. 2. 3D imaging of eDNA (green) and total nucleic acids (red), for hospital and urban *in-situ* and *in-vitro* biofilms. Autofluorescence for unknown compounds is in blue. +Cip= *in vitro* biofilms were exposed after 3 days of initial biofilm formation with 1 $\mu\text{g}/\text{ml}$ ciprofloxacin for 4 consecutive days and harvested after exposure of 4 h on the final day. .

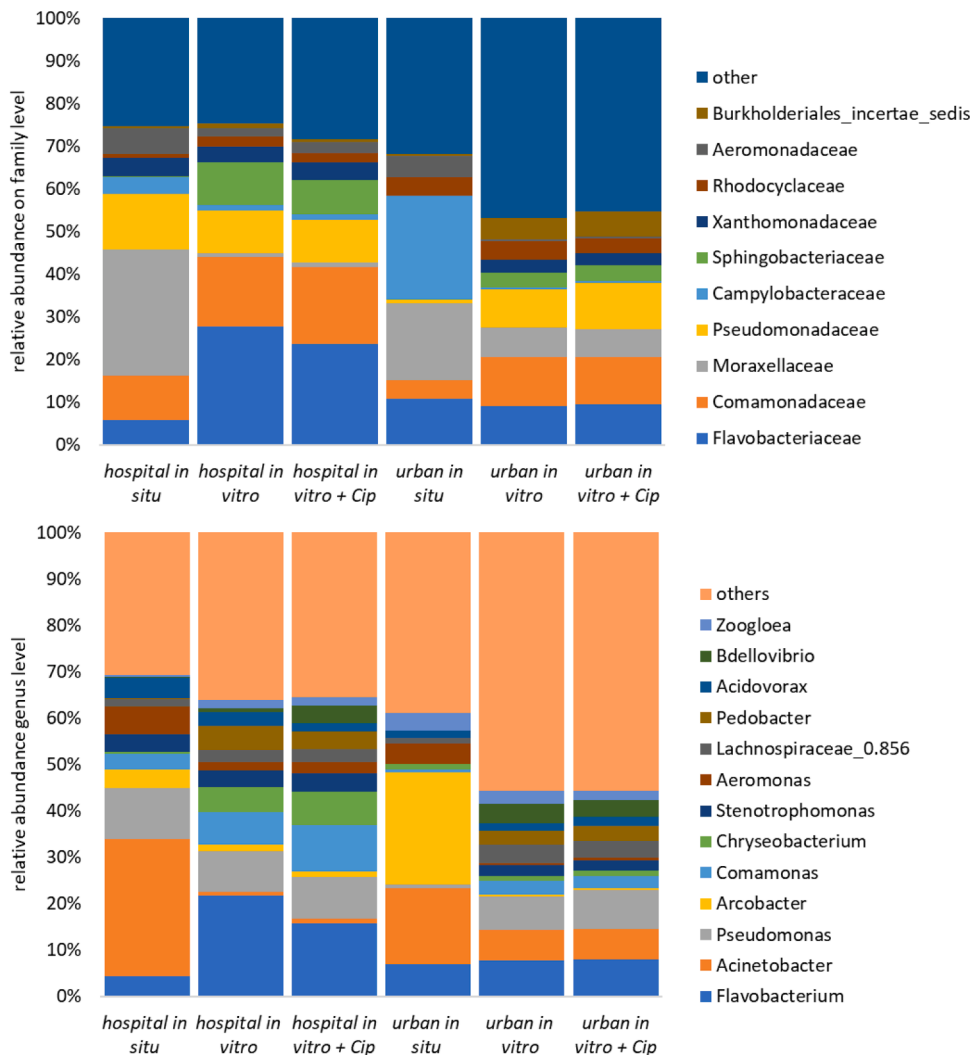


Fig. 3. Bacterial community composition of hospital and urban wastewater *in situ* and *in vitro* biofilms based on sequencing of the 16S rRNA gene. Depicted are the top 10 taxa on order level and the top 12 on the genus level, the remaining taxa are grouped into others. Unknown reads not assigned on SILVA range between 1.7% and 13.5% on order, and between 9.8% and 28.1% on genus level, respectively and are included into others. +Cip= *in vitro* biofilms were exposed after 3 days of initial biofilm formation with 1 $\mu\text{g}/\text{ml}$ ciprofloxacin for 4 consecutive days and harvested after exposure of 4 h on the final day (D7).

diversity, whereas the urban *in vitro* biofilms had the highest diversity (Supplementary Figure 4 b). To compare the diversity and microbiota composition between the samples, we calculated the proportion of the Simpson's diversity between samples (Supplementary Figure 4 a), which takes not only presence and absence of taxa into account, but also their relative abundance. Here we showed that the addition of ciprofloxacin to the WW in our *in vitro* model did not change the proportional microbiota composition for hospital and urban biofilms (Fig. 3, Supplementary Figure 4) on the order level. However, on genus level, the addition of ciprofloxacin had a significant impact on the diversity of the microbiota of the *in vitro* hospital WW biofilm (Fig. 3 and Supplementary Figure 4 a).

As described in methods (supplementary data), a relatively large proportion, between 42% and 70%, of the total RNA reads from the metatranscriptomic RNA sequencing approach of the biofilm communities were transcripts of ribosomal RNA (rRNA) reads. These 16S rRNA reads were filtered out by mapping them on the Silva database, allowing for the taxonomic assignment of a small proportion of those reads (between 0.68% and 12%) on family level. It is important to note that the taxonomy composition extrapolated from rRNA reads obtained from RNA sequencing after *in vitro* and *in silico* rRNA depletion is not directly correlated to the microbial community diversity. However, it may be indicative of protein synthesis potential of the respective bacterial families, independent of their metabolic state or live bacteria (Blazewicz et al., 2013; Li et al., 2017). Only those rRNA reads with at least 10,000 reads classifiable on family level in at least one sample were

assigned and considered, likely representing bacterial families with higher protein synthesis potential, hence metabolic activity, within these complex biofilm communities. Among the classifiable 16S rRNA reads from the metatranscriptomic analysis, we identified a large majority of *Enterobacteriaceae* in hospital biofilms, a mix of *Moraxellaceae*, *Flavobacteriaceae*, *Enterobacteriaceae*, *Streptococcaceae* and *Francisellaceae* in urban *in situ* biofilm, and a very high proportion of *Lactobacillaceae* for the *in vitro* urban biofilm control (Supplementary Figure 5 and Supplementary Table 3).

3.3. The targeted resistome and its expression in hospital and urban WW biofilms

To assess the resistome in our samples, a targeted high-throughput qPCR approach, that detects and quantifies 86 individual genes conferring resistance to clinically relevant antibiotics, quaternary ammonium compounds (QACs), heavy metals, transposase genes (MGEs) and integron integrase genes, also referred to as targeted resistome, was applied. At first, comparing the different environments in which biofilms grew (*in situ* vs *in vitro*), we found that for the hospital WW biofilms, all gene classes had a higher normalized abundance *in situ* biofilm compared to the *in vitro* biofilms, except for aminoglycosides, quinolones, QACs, sulfonamides, MGEs and integrons, which were not significantly different (Fig. 4A, Supplementary Figure 6, and Supplementary Table 4). Performing the same comparison for the urban WW *in situ* vs the *in vitro* biofilms, the normalized abundances of

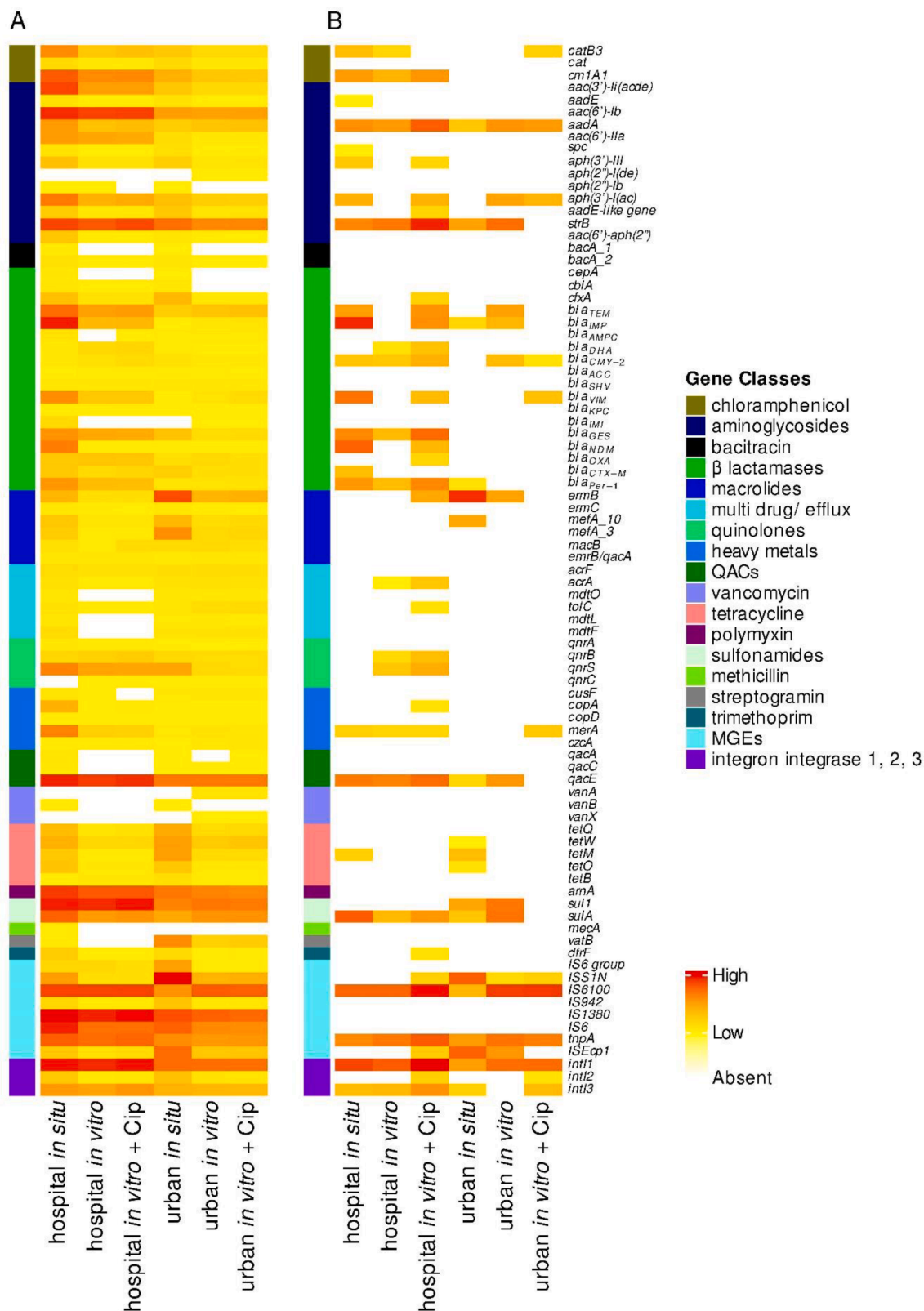


Fig. 4. The targeted resistome and its expression in hospital and urban WW biofilms. Heatmaps depicting (A) the log₂ of normalized abundance of individual genes (targeted resistome) by qPCR and (B) their normalized expression levels (exact counts of mapped reads for the respective genes) for the individual genes detected by metatranscriptomics (RNA sequencing). +Cip= *in vitro* biofilms were exposed after 3 days of initial biofilm formation with 1 μ g/ml of the antibiotic ciprofloxacin for 4 consecutive days and harvested after exposure of 4 h on the final day (D7).

chloramphenicol, macrolides, quinolones and MGEs, were significantly higher for the *in situ* biofilms, whereas genes conferring resistance to heavy metals, vancomycin, and sulfonamides had significantly lower normalized abundances *in situ* urban WW biofilms. Then, comparing the different sampling origin (untreated hospital WW vs urban WW), the normalized abundance of the targeted resistome was highest in the *in situ* hospital WW biofilms for all detected gene classes apart from macrolides, which were higher in the urban *in situ* biofilm, while tetracyclines, quinolones and MGEs, were not significantly different in their normalized abundance between the two *in situ* biofilm sampling origins (Supplementary Figure 6, and Supplementary Table 4). Comparing the normalized abundance of detected gene classes for the hospital vs urban *in vitro* biofilms, it was higher in the hospital *in vitro* biofilms for most genes, except for macrolides, vancomycin and genes encoding for multi-drug efflux pumps for which the normalized abundance was higher in the urban *in vitro* biofilms; no significant difference was observed for tetracyclines and heavy metal resistance genes.

Then, we analysed the metagenomic messenger RNA (mRNA) to provide information on the targeted resistome actively expressed in the biofilms. Overall, 39 of the 86 targeted genes by qPCR were expressed in at least one of the samples (Fig. 4B). Comparing first the biofilm environments (*in situ* vs *in vitro*), the expression level of beta-lactam, chloramphenicol and sulfonamide resistance genes was respectively 112.6, 5, and 47.5-fold higher in the hospital *in situ* biofilm vs hospital *in vitro* biofilm, whereas the expression level of macrolide resistance genes was 49.1-fold higher in the urban *in situ* biofilm vs the urban *in vitro* biofilm, and for all other classes of genes whose expression could be detected, the detection levels were lower in urban *in situ* biofilm vs urban *in vitro* biofilm (Fig. 4B, Supplementary Table 5). Considering then the biofilm sample origins (untreated hospital WW vs urban WW), expression levels were higher for 6 gene classes, from 5.5- to 957.5-fold for genes conferring resistance to aminoglycosides and beta-lactams respectively, (Supplementary Table 5) for hospital *in situ* vs urban *in situ* WW biofilms.

We studied the impact of ciprofloxacin in the *in vitro* biofilm models. Interestingly, when analyzing mRNA, we observed an overall enhanced expression of the targeted resistome (Fig. 4B, Supplementary Table 5) after ciprofloxacin addition in the hospital *in vitro* biofilm (sign test, $n = 9$, p -value = 0.021). For example, expression levels of ARGs conferring resistance to beta-lactams and aminoglycoside resistance genes was on average 25.7-fold and 10.5-fold higher, respectively and represented the classes of ARGs that were most abundantly expressed proportionally (Fig. 4b, Supplementary Table 5 and Supplementary Figure 7). On the contrary, expression levels of the targeted resistome in the urban *in vitro* biofilm exposed to ciprofloxacin, was lower for all detected gene classes apart from the integron integrase genes, whose expression was slightly increased by 1.1-fold respectively, compared to its control (Fig. 4B, Supplementary Table 5). The *ISS1N* gene, a member of the IS6 family, common in Gram-positive lactic acid bacteria (Haandrikman et al., 1990), was specifically expressed in the *in situ* urban WW biofilm (Fig. 4B).

Overall, 45% of the targeted resistome detected by qPCR was expressed in the WW biofilms based on our complementary RNA-based metatranscriptomic approach. The expression of ARGs and integron integrase genes in the *in vitro* hospital WW biofilm was enhanced by the addition of ciprofloxacin. Using binomial model prediction and linear regression analysis, we investigated the likelihood of genes that were quantified by qPCR (DNA-based method) to be detected by RNA sequencing and metatranscriptomics. Genes that were the most abundant in the targeted resistome (qPCR) had a higher probability of being detected by metatranscriptomics. At a threshold of 0.057 for the normalized abundance of genes in the targeted resistome, the probability of being detected by metatranscriptomics is 0.5. Below this threshold, the detection of ARG expression by metatranscriptomics could no longer be achieved, suggesting a lack of transcripts. Furthermore, for the genes that were detected by both approaches (DNA-based and RNA-based), we observed a positive correlation between the two

quantifications. (Rho=0.77; Supplementary Figure 8 and Supplementary Table 6). However, the technical limitations of meta-transcriptomics due to low sequencing depth and high rRNA levels and/or the detection of dead cell's DNA and eDNA by qPCR could also explain why the meta-transcriptomic approach did not detect a larger number of targeted genes (Batovska et al., 2019; Eramo et al., 2019; Sivalingam et al., 2020).

3.4. Assessing the expression of ARGs based on the CARD database

To identify additional ARGs that were not included in our targeted resistome approach, mRNA reads of our samples were mapped against the Comprehensive Antibiotic Resistance Database (CARD) that archives thousands of gene sequences encoding resistance determinants to antimicrobial agents (see supplemental data for details).

ARG expression was higher for *in situ* hospital WW biofilms compared to their *in vitro* counterpart (10.4% and 0.5% of total mRNA reads respectively) (Supplementary Table 7), and lower in the *in situ* urban WW biofilms compared to their *in vitro* counterpart (respectively 0.04% and 0.4%; supplementary Table 7). A 260-fold higher expression level of ARGs was detected in the *in situ* hospital WW biofilm (10.4% of all mRNA reads coding for ARGs) compared to the urban WW *in situ* biofilm (0.04% of mRNA reads coding for ARGs) (Supplementary Table 7). Exposure to ciprofloxacin increased the level of ARGs expression in the hospital *in vitro* biofilm by 4.4-fold (2.4% of mRNA reads + Cip vs 0.55% when untreated, Supplementary Table 7), but had no significant effect on the level of ARG expression in urban *in vitro* biofilms (0.37% vs 0.41% of total mRNA reads mapping to ARGs Cip treated and untreated respectively, Supplementary Table 7).

In total we detected the expression of 70 individual resistance genes conferring resistance to 15 classes of antibacterial drugs across all biofilm samples (Fig. 5, Supplementary Table 8). This approach led to the detection of the expression of additional genes in hospital and urban WW biofilms that were not detected by the targeted approach (Supplementary Table 8). For example, active beta-lactamase such as *bla_{RCP}*, *bla_{LCR}*, *bla_{FOX}*, *bla_{NPS}*, four aminoglycoside-modifying enzymes (*aac(3)-Ib/aac(6)-Ib*, *aadB*, *aph(3')*, *aph(6)*) and a macrolide phosphotransferase (MPH) were identified. Genes that confer resistance to the antimicrobial classes fusidic acid, elfamycin, aminoglycosides, beta lactamases- amongst them *bla_{OXA}*, sulfonamides, macrolides and multi drug efflux pumps, were found to be expressed in all the investigated samples. Genes that confer resistance to isoniazid and triclosan were found to be expressed in the *in vitro* hospital biofilm exposed to ciprofloxacin, and *in vitro* urban WW biofilms in both conditions. The expression of an enterobacterial fosfomycin resistance gene (*ptsI*) was detected in the *in vitro* hospital WW biofilm exposed to ciprofloxacin (Supplementary Table 8).

Overall, the composition of the ARG meta-transcriptome of the CARD resistome analysis correlated to the targeted resistome analysis (Supplementary Figure 4a, Supplementary Tables 5 and 9), with additional ARGs detected as described above. Comparing the different environments for hospital biofilms (*in situ* vs *in vitro*), higher gene expression levels were detected for the *in situ* hospital biofilm, between 3.5-fold and 15.2-fold (macrolides and beta-lactamases respectively, Supplementary Table 9). For the *in situ* vs *in vitro* urban biofilms, expression levels were lower for all detected ARG classes (Supplementary Figure 9, Supplementary Tables 8 and 9). When hospital *in vitro* biofilm exposed to ciprofloxacin was compared to its control, an overall increase in expression level was detected for eight ARG classes, between 1.3- and 7.5 times, for genes encoding fusic acids and genes conferring resistance to beta-lactams, respectively. Four of these gene classes were significantly more expressed compared to the non-ciprofloxacin exposed control (aminoglycosides, beta-lactamases, fluoroquinolones and multi drug efflux pumps, Supplementary Figure 9, and Supplementary Table 9). For the urban *in vitro* biofilm exposed to ciprofloxacin, expression levels were lower, except for genes encoding beta-lactams

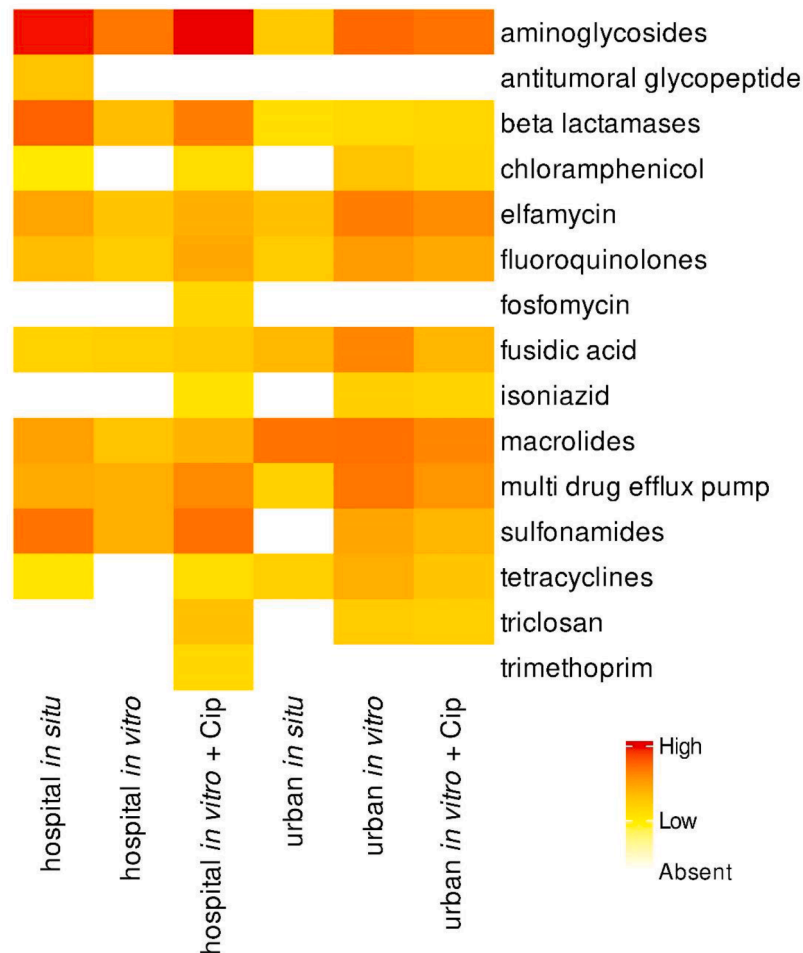


Fig. 5. ARG metatranscriptome based on the CARD database. Heatmap of mean numbers of mapped reads per antimicrobial resistance gene were summed by ARG class and are displayed per sample.

with a slight increase (1.18-fold) compared to its control (Supplementary Table 8 and 9).

3.5. The active resistance mobilome

To study the expression of MGEs and more specifically MGEs associated with ARGs, assembled contigs resulting from the RNA-sequencing approach, were mapped against the plasmid sequence collection v0.4 available at the ACLAME database (Lepplae et al., 2010). Subsequently, the contigs associated with plasmid identified by this approach were mapped back to the CARD database to specifically identify plasmids carrying ARGs. These ARG-associated plasmids are referred to as the “active resistance mobilome” in the respective samples.

Global expression of the active resistance mobilome was very low in all the samples studied (0.48% of all assembled contigs for hospital *in situ* biofilm and 0.14% for urban *in situ* biofilm (Supplementary Table 7). In total, we counted respectively 115 and 66 ARG-associated plasmid contigs for the hospital and urban *in situ* biofilms and identified a diverse range of plasmid genes (71 individual plasmids identified across all samples) expressing a diverse range of ARGs that could be grouped into 10 classes conferring resistance to antimicrobials, quaternary ammonium compounds and heavy metals. The highest number of ARG-associated plasmids (50) was detected in the *in vitro* hospital biofilm exposed to ciprofloxacin (Supplementary Figure 10).

Overall, 56 different plasmids carrying a broad range of ARGs were identified as the active resistance mobilome across all hospital WW biofilm samples. These plasmids were mainly resistance plasmids found

in Gram-negative pathogens such as the bla-VIM7 carrying plasmid pmATVIM-7 first identified in *P. aeruginosa* (Toleman et al., 2004); the IncP-6 plasmid *Rms149*, a small resistant plasmid associated with *P. aeruginosa* (Haines et al., 2005) (detected only in hospital *in situ* biofilm); several small resistance plasmids (e.g. *pKMA757*, *pARD3079*, *pKMA202*, and *pKMA5*) associated with Actinobacilli (Matter et al., 2008) and plasmid families representing the groups IncP, F, N and Q (Supplementary Figure 10). They primarily carried aminoglycoside, sulfonamide, chloramphenicol, and beta-lactam resistance genes (Fig. 6; Supplementary Figure 10). Fourteen plasmids were detected in all hospital WW biofilm samples, and 7 additional plasmids were only shared between the *in situ* hospital WW biofilms and the *in vitro* hospital WW biofilm exposed to ciprofloxacin (Supplementary Figure 10). Interestingly, 22 plasmids, carrying between 1 and 5 ARGs, were only identified in the *in vitro* hospital WW biofilm exposed to ciprofloxacin (Supplementary Figure 10).

Among the total 71 ARG-associated plasmids, 46 were detected across all urban WW biofilm samples, 14 of which were unique to urban WW biofilm samples (*in situ* and *in vitro*) (Supplementary Figure 10). These plasmids mainly carried macrolide and tetracycline resistance genes found in Gram-positive bacteria, such as Lactobacilli. In addition, the *Enterococcus faecium* and *faecalis* plasmids *pRUM* and *pTEF1* have been identified as carriers of the macrolide resistance gene *ermB* (Jensen et al., 2010, 1999; Palmer et al., 2010) (Supplementary Figure 10). The broad host range IncP-1 plasmid pKJK5 was also detected, carrying an aminoglycoside (*aadA6*) and a sulfonamide (*sul1*) resistance gene (Popowska and Krawczyk-Balska, 2013; Røder et al., 2013). One

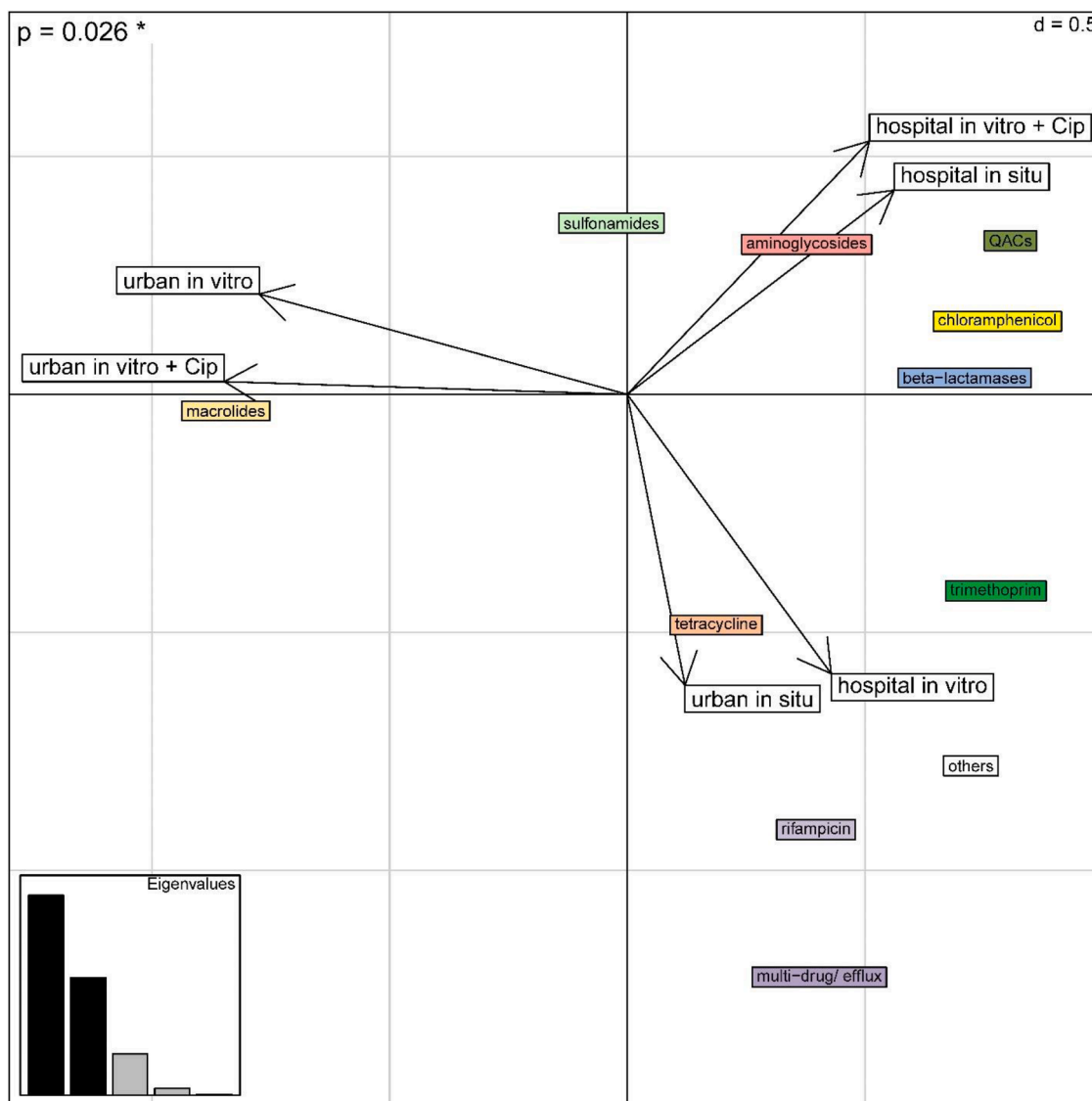


Fig. 6. Between correspondence analysis (bCA) assessing the association between the type of ARGs classes present on plasmids, in terms of ARG class abundance (numbers of contigs) and the biofilm origin.

Gram-negative multidrug resistance plasmid *pJR1* (Wu et al., 2003) was detected, carrying the chloramphenicol resistance gene *catB2* and the tetracycline resistance gene *tet(G)* (Supplementary Figure 10). Twenty-eight plasmids were uniquely identified in the *in situ* urban WW biofilm and not in its *in vitro* counterpart. Six of these plasmids were also detected in the hospital *in situ* WW biofilm (e.g. *pJHCMW1* and *R46*) (Supplementary Figure 10).

In hospital WW biofilms, the majority of ARG-associated plasmids were identified to carry multiple resistance genes (up to 5 ARGs carried on one MGE contig, Supplementary Figure 10). These genes mainly encoded resistance to aminoglycosides, beta-lactams, sulfonamides, chloramphenicol, and quaternary ammonium compounds (Fig. 6, Supplementary Figure 10). For urban WW biofilms, ARG-associated plasmids carrying multiple resistance genes were also identified, but with less ARGs associated (up to 3 ARGs on one plasmid in the *in situ* urban WW biofilm) (Supplementary Figure 10). The ARGs mainly detected in the active resistance mobilome of urban WW biofilms were macrolides, tetracyclines and multi-drug efflux pumps (Fig. 6, Supplementary Figure 10). In the *in vitro* urban biofilm exposed to ciprofloxacin only 9 ARG-associated plasmids could be identified (mainly macrolide resistance genes) (Fig. 6, Supplementary Figure 10).

Overall, correspondence analysis (CA) of our active resistance mobilome data revealed a prominent association between ARGs present on plasmids and their biofilm origin (Cramér's V statistic = 0.604; χ^2 -test p -value < 0.001; Supplementary Figure 11). To assess if this association was also observed at the level of the ARGs classes, we applied a between CA (bCA). This recovered 33% of the total association between biofilm origin and ARGs composition of the active resistance mobilome (p -value=0.026; Fig. 6).

4. Discussion

The objective of this study was to assess the capacity of WW biofilms as putative hotspots that favor transfer of ARGs via MGEs within these bacterial communities. To do this, we implemented a comprehensive experimental approach applied to WW biofilms of different origins (hospital WW and urban WW) and grown in different environments (*in situ* and *in vitro*), focusing on four dimensions of the multispecies WW biofilms, namely, extracellular polymeric substances (EPS), community composition, and presence and activity of ARGs and MGEs. Given the proposed involvement of EPS, specifically eDNA, in horizontal gene transfer within biofilms, as well as their role in antibiotic tolerance and

response to external stressors (Hannan et al., 2010; Karygianni et al., 2020; Soares et al., 2019), we quantified and visualized the major EPS components of these biofilms. In addition, unlike traditional methods that focus mostly on the presence and relative abundance of ARGs, we assessed both the presence of ARGs and MGEs (DNA-based) as well as their expression and activity (RNA-based).

Our study provides a comprehensive assessment of WW biofilms as crucial hotspots for ARG transfer. Hospital WW biofilms exhibited distinct characteristics, including higher eDNA abundance and expression levels of ARGs and MGEs, highlighting their role in antimicrobial resistance dissemination.

We implemented a spectral imaging method using confocal laser scanning microscopy (CLSM) to visualize and quantify the EPS of biofilms. Our findings revealed that the abundance of the investigated EPS components differed depending on the growth environment (*in situ* and *in vitro*) and the origin of sampling (hospital vs. urban WW). Moreover, exposure to ciprofloxacin at its minimal selective concentration (MSC) resulted in a decrease in the relative abundance of polysaccharides and eDNA in both urban and hospital *in vitro* biofilms. This observation could be attributed to a regulatory response by the biofilm community, as previous studies have demonstrated reduced EPS production in multi-species biofilms under exogenous stress (Loustau et al., 2021). Notably, the abundance of eDNA was relatively higher in hospital WW biofilms compared to urban WW biofilms, potentially due to elevated levels of surfactants, antibiotics, and other pharmaceutical and chemical pollutants in hospital WW (Buelow et al., 2020). These factors may promote bacterial lysis and active eDNA secretion within hospital WW biofilms (Okshevsy and Meyer, 2015). eDNA has been shown to confer antimicrobial tolerance in biofilms by impeding the diffusion of cationic molecules (Wilton et al., 2015) and enhancing resistance to aminoglycosides in *Pseudomonas aeruginosa* biofilms (Doroshenko et al., 2014; Lewenza, 2013). Furthermore, eDNA has been implicated in the transfer of the ARG *tetM* through the conjugative transposon Tn916 (Hannan et al., 2010) via transformation. Transformation is thought to be an important mechanism for gene transfer between bacterial communities in biofilms (Hannan et al., 2010) and maybe correlated with the abundance of eDNA in biofilm. The distinct microbiota composition *in situ* hospital and urban WW biofilms likely contributes to the differences in organization and relative abundances of EPS components in these two biofilm niches. However, further longitudinal studies are needed to investigate the extent to which environmental changes, such as high exogenous stress or shifts in microbial composition, impact the EPS and the actual role of eDNA in HGT within natural and complex biofilm communities. These future studies will provide valuable insights into the relationship between eDNA as a structural component within the biofilm matrix, its active role, and its significance as a reservoir for horizontal gene transfer in natural biofilm communities.

In our study, we employed a metatranscriptomic approach to achieve three main objectives. Firstly, we aimed to assess the baseline expression levels of ARGs-associated MGEs (active resistance mobilome) as an indicator of the actual transfer of ARGs within natural, *in situ*, hospital and urban WW biofilms. Secondly, we sought to compare these expression levels with those observed *in vitro* biofilms. Finally, we investigated whether these baseline expression levels changed in response to the addition of ciprofloxacin at MSC to the *in vitro* WW biofilm environment.

Our findings revealed several important observations. First, we found that the basal expression levels of ARGs and MGEs were higher *in situ* WW hospital biofilms compared to *in vitro* biofilms. This suggests that the natural biofilm environment and its origin (WW type) have a significant influence on the expression of ARGs and MGEs. Second, upon the addition of a MSC of ciprofloxacin to the *in vitro* hospital WW biofilm, we observed an increase in the expression levels of both ARGs and MGEs. This indicates that antibiotic exposure of the SOS-response inducer ciprofloxacin at MSC can induce the upregulation of resistance mechanisms within biofilms. Third, we observed that the characteristics of the active resistance mobilome, such as specific classes of ARGs and

the number of ARG-associated plasmids, were correlated with the origin of the biofilm. This suggests that the content of ARGs on plasmids is adapted to the specific biofilm environment.

Another notable finding was that the microbiota and EPS composition of the *in vitro* biofilms from hospital and urban WW were more similar to each other than to their *in situ* counterparts. This suggests that the laboratory conditions used in our *in vitro* model have a stronger influence on the structure of the biofilm community than the original WW environment in which they developed. This raises concerns about the ability of the *in vitro* model to accurately mimic the complexities of the natural environment and highlights the need for further optimization to improve its representativeness.

Indeed, the sequencing depth and robustness of our metatranscriptomic approach are crucial factors to consider in interpreting our results. We encountered challenges in RNA extraction and rRNA depletion, which impacted the number of samples we could sequence and the efficiency of rRNA removal from the biofilm samples. These limitations emphasize the need for further optimization of RNA extraction methods and the exploration of alternative rRNA depletion techniques in future studies.

Despite the challenges, we were able to assess the quality of our mRNA assembly and analysis by utilizing *in silico* rRNA depletion. This approach improved the accuracy and reliability of our results, allowing us to draw meaningful conclusions regarding the presence MGEs and ARGs in the biofilms. The use of a kmer length of 21 for assembly optimization and stringent analysis cut-offs further ensured the robustness of our findings.

Moreover, the analysis of rRNA-based community composition after rRNA depletion provided valuable insights into the protein synthesis potential of microbial communities within the biofilms. Although rRNA levels cannot be interpreted quantitatively due to differential depletion efficiency and potential misleading information in complex biofilm communities, they serve as an indicator of the protein synthesis activity within the biofilms.

Analyzing the classifiable rRNA reads, we identified a predominance of *Enterobacteriaceae* in hospital biofilms and a mixture of *Moraxellaceae*, *Flavobacteriaceae*, *Enterobacteriaceae*, *Streptococaceae*, and *Francisellaceae* in urban *in situ* biofilms. Surprisingly, *Lactobacillaceae* was the most proportionally abundant taxa in the urban *in vitro* control biofilm. While this may be an artifact introduced by rRNA depletion biases, it may also indicate the presence of highly "active" taxa in the bacterial community (Blazewicz et al., 2013; Li et al., 2017). By focusing on the active community, which includes taxa that are actively transcribing and producing proteins, we can better assess their role in antibiotic resistance dynamics. Taxa that are highly active, even if they are not very abundant based on DNA-based analysis, may still play a significant role in resistance gene expression and dissemination within the biofilm. Therefore, studying the active biofilm community provides valuable insights on the functional aspects of antibiotic resistance and on the dynamics of resistance gene transfer.

In previous longitudinal studies, urban WW has been characterized by a high abundance of macrolide ARGs and Gram-positive/environmental bacteria (Buelow et al., 2020; Karkman et al., 2016). In line with these findings, we detected macrolide ARGs and plasmids associated with Gram-positive lactobacilli more frequently in urban biofilm samples, suggesting a correlation between the expression of specific ARGs and microbial composition. This supports the notion that the active proportion of *Enterobacteriaceae* in hospital biofilms may explain the higher expression levels and types of MGEs and ARGs detected in these samples, underlining the role of human gut-associated bacteria and fecal pollution in the environment, in the dissemination of antibiotic resistance (Karkman et al., 2019; Buelow et al., 2020; McInnes et al., 2021).

Furthermore, our study identified distinct plasmid profiles and specific ARG expression profiles for hospital and urban WW biofilms, indicating strong environmental selection pressure shaping the active

resistance mobilome. These findings are consistent with a recent study that showed that most plasmid-borne ARGs, including those localized on class-1 integrons, are enriched in conjugative plasmids, and that transfer between AMR plasmids and bacterial chromosomes is mediated by insertion sequences, particularly those belonging to the IS6 family, IS26, and IS6100 (Che et al., 2021). The abundance of IS26 and IS6100 in WW (Buelow et al., 2020) and their high expression in hospital WW biofilms in this study suggest their potential role in facilitating the expression of ARGs.

In summary, our study highlights the importance of studying the active biofilm community and its resistance mobilome. The higher expression levels and specific types of MGEs and ARGs observed in hospital biofilms, along with their response to antibiotic stress, suggest that human-gut associated bacteria, particularly *Enterobacteriaceae*, play a significant role in the accumulation and transfer of ARGs in highly anthropized environments such as wastewater. However, it is essential to consider alternative hypotheses, such as the possibility of interactions between ciprofloxacin and biofilm components that impede the activity of ciprofloxacin within the respective biofilms. These aspects should be further investigated to deepen our understanding of the complex dynamics within biofilm communities.

The positive correlation between DNA-based and RNA-based quantifications underscores the importance of examining gene expression and activity in addition to their mere presence. This dynamic perspective allows us to gain a deeper understanding of the functional relevance of antibiotic resistance genes within biofilm communities. By considering both the potential for gene transfer through MGEs and the active expression of these genes, we can more accurately assess the role of biofilms as reservoirs and hotspots for the spread of antibiotic resistance.

5. Conclusions

- *In situ* biofilm matrix composition is influenced by the growth environment and microbiota.
- Hospital and urban wastewater biofilms differ in their potential to disseminate ARGs through MGEs, highlighting hospital WW biofilms as important hot spot for the dissemination and selection of AMRs.
- Active *Enterobacteriaceae* in hospital WW biofilms likely correlate with high expression of ARGs and MGEs, highlighting the role of human gut bacteria and fecal pollution in AMR dissemination.
- Hospital WW biofilms offer an ideal model to study the impact of pollution and human gut bacteria on AMR dissemination and biofilm organization.
- Understanding AMR dynamics in hospital WW biofilms can contribute to the development of strategies to combat AMR and to mitigate environmental risks.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors gave their consent for publication.

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Authors' contributions

C.D., S. D.-R., M.-C.P., and E.B. designed the study, Ca. D. and S.A. developed the metatranscriptomic analysis pipeline, M.G. and E.B. performed experiments, E.B., Ca.D., H.M.H., T.J. and S.P.K. performed

data analysis, O.C. and H.M.H provided critical comments on the content of the manuscript, E.B., wrote the manuscript with contribution of all other co-authors. All authors reviewed the manuscript.

Declaration of Competing Interest

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

Data availability

16S rRNA sequence data are available at the European Nucleotide Archive (ENA) under the accession numbers ERS14475391–8. RNA sequencing data (metatranscriptomic data) will be available at the NCBI under project number PRJNA930611. All other important raw data needed to reconstruct the findings of our study are made available in the supplementary material.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2023.120408](https://doi.org/10.1016/j.watres.2023.120408).

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