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Dynamics of antibiotic resistance markers and *Escherichia coli* invasion in riverine heterotrophic biofilms facing increasing heat and flow stagnation

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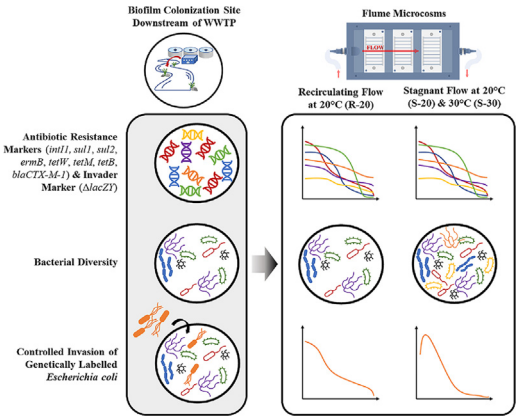
HIGHLIGHTS

- Flume experiment shows that river biofilms downstream of wastewater effluent are invadable by *E. coli*.
- *E. coli* invasion is transient regardless of varying environmental conditions.
- Neither stagnancy nor high temperature increases persistence of antibiotic resistance markers.
- Antibiotic resistance markers decrease in biofilm with no external stressors.
- Microbial community composition in river biofilm diversifies with flow termination.

GRAPHICAL ABSTRACT

Visual abstract: Experimental design and key results



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ABSTRACT

As motivation to address environmental dissemination of antimicrobial resistance (AMR) is mounting, there is a need to characterize mechanisms by which AMR can propagate under environmental conditions. Here we investigated the effect of temperature and stagnation on the persistence of wastewater-associated antibiotic resistance markers in riverine biofilms and the invasion success of genetically-tagged *Escherichia coli.* Biofilms grown on glass slides incubated in-situ downstream of a wastewater treatment plant effluent discharge point were transferred to laboratory-scale flumes fed with filtered river water under potentially stressful temperature and flow conditions: recirculation flow at 20 °C, stagnation at 20 °C, and stagnation at 30 °C. After 14 days, quantitative PCR and amplicon sequencing were used to quantify bacteria, biofilms diversity, resistance markers (*sul1, sul2, ermB, tetW, tetM, tetB, blaCTX-M-1, intI1*) and *E. coli.* Resistance markers significantly decreased over time regardless of the treatment applied. Although invading *E. coli* were initially able to colonize the biofilms, its abundance subsequently declined. Stagnation was associated with a shift in biofilm taxonomic composition, but there was no apparent effect of flow conditions or the simulated river-pool warming (30 °C) on AMR persistence or invasion success of *E. coli.* Results however indicated that antibiotic resistance markers in the riverine biofilms decreased under the experimental conditions in the absence of exposure to external inputs of antibiotics and AMR.

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1. Introduction

Since their discovery in the 20th century, antibiotics have come to serve as one of the most common means of treating bacterial infections in animals and humans (European Commission, 2017; United Nations Environment Programme, 2022). With the rise in antimicrobial resistance (AMR) and antibiotic resistant bacteria (ARB), common antibiotics are losing efficacy and once treatable diseases are becoming increasingly life-threatening (The Swiss Federal Council, 2015). Worldwide, roughly 700,000 annual deaths have been linked to AMR with 25,000 in the European Union (European Commission, 2017). Infections and deaths from drug-resistant, hospital-acquired bacteria rose by 15 % from 2019 to 2020 in the US, with alarming increases in some of the most highly resistant bacterial pathogens including carbapenem-resistant *Acinetobacter baumannii* and *Enterobacterales* and multidrug-resistant *Pseudomonas aeruginosa*, among others (Centers for Disease Control and Prevention; National Center for Emerging and Zoonotic Infectious Diseases; Division of Healthcare Quality Promotion, 2022). In addition to contributing to direct health risks, AMR poses a significant economic burden, with an estimated $ 1.5 billion loss in annual productivity within the EU alone. As a result, many governments consider AMR a major global public health concern (European Commission, 2017).

Antibiotics, antibiotic resistance genes (ARGs), and ARB can persist and proliferate in the natural environment through a variety of mechanisms, such as selection under exposure to sub-inhibitory concentrations of antibiotic, vertical gene transfer, and horizontal gene transfer (HGT) (Jutkina et al., 2018; Sanchez-Cid et al., 2022; Singh et al., 2019). Although a spectrum of resistance determinants can be found in remote or low impact environmental microbiomes (e.g., transferred vertically over generations, receiving inputs form wild animals and atmospheric deposition (Gao et al., 2022; Jarma et al., 2021; Kormos et al., 2022; Vittecoq et al., 2016)), the release and reuse of wastewater effluent and animal farming residues are known to be the primary conduits of AMR spread in the aquatic context (Flores-Vargas et al., 2021). HGT involves the exchange of genetic information between bacteria through transduction, transformation, or conjugation (Soucy et al., 2015). Bacteria that acquired AMR through HGT may attain an evolutionary advantage and increased fitness compared to susceptible bacteria in environments experiencing selective pressures, such as increased antibiotic exposure in hospital effluent and wastewater treatment plants (Ju et al., 2018; Vaz-Moreira et al., 2014). Environmental matrices, such as river water (Lee et al., 2021; Marti et al., 2013), sediment (Reichert et al., 2021), and biofilms (Guo et al., 2018; Reichert et al., 2021), are thought to be reservoirs and conduits of AMR dissemination. Waste streams with elevated resistance levels such as wastewater, hospital waste and pharmaceutical industry effluents alter the *resistome* (i.e. collective ARGs in a microbial community) of such matrices and create favorable conditions for the AMR determinants to proliferate and persist (Bielen et al., 2017; Hocquet et al., 2016; Singh et al., 2019). In rivers, Lee et al. (2021) determined that certain ARGs persisted in water over long distances downstream of wastewater treatment plant (WWTP) discharge points. Similarly, Proia et al. (2016) found significantly higher *sul1* and *ermB* gene abundances persisting in biofilms downstream of WWTP effluent. Balcázar et al. (2015) not only determined that ARGs persisted downstream of WWTP, but also that their relative abundance in biofilms increased significantly over distance. It is less clear, however, whether ARGs will persist in the absence of wastewater inputs, typically rich in ARBs, antibiotics or other selective agents. Under real-world conditions, the environmental pressures, such as industrial contamination, wastewater effluent discharges, intensive agriculture procedures, and residues of antimicrobial compounds present in feces and urine inputs may influence the ARGs persistence (Larsson and Flach, 2022). For instance, metals, pharmaceutical and antibacterial biocides can, in many cases, accelerate rates of HGT (Klümper et al., 2017; Pal et al., 2017) and co-select through co-resistance (via genetically linked mechanisms) or cross-resistance (via the same gene) for antibiotic-resistant strains (Aminov, 2011; Larsson and Flach, 2022).

Biofilms within the river biome are thought to be a primary facilitator of AMR proliferation in aquatic systems (Abe et al., 2020). Due to the close proximity of cells in the extracellular matrix as well as high cell population densities, biofilms are thought to be a conducive environment for elevated HGT compared to planktonic bacteria, and thus harbor higher levels of ARG exchange and persistence (Balcázar et al., 2015; Guo et al., 2018; Molin and Tolker-Nielsen, 2003). Recently, Li et al. also found that the invasion by multidrug resistant *Escherichia coli* of a microbial community prior to antibiotic exposure, increased the levels of vertical and horizontal gene transfer, and thus promoted the dissemination of ARGs carried across the microbial community (Li et al., 2021). Invasion of river biofilms by multidrug bacteria from other sources, e.g. wastewater, could accordingly be a key initial step for HGT of ARGs in biofilm communities. Several studies have demonstrated that *E. coli* is one of the most important indicators of both fecal pollution and ARGs of anthropogenic origin (Devarajan et al., 2016; Nowicki et al., 2021; Zhang et al., 2020). Because *E. coli* exhibits large genotypic and phenotypic diversity across strains, it is able to contend with many stresses and populate many environmental habitats; including lakes, rivers, sediments, beaches, soils, and both treated water and wastewater (Berthe et al., 2013; Ferguson et al., 2011; Ishii and Sadowsky, 2008). Consequently, *E. coli* has been suggested as a potential indicator organism to monitor AMR in environmental settings (Berendonk et al., 2015). Here we used the non-resistant *E. coli* strain, CM2372, containing a *ΔlacZY* gene tag that enables its detection within a natural community. Merlin and colleagues previously released the strain to be used as a tagged model invader to study environmental mechanisms of AMR dissemination (Merlin et al., 2002). As the temperature optimum of *E. coli* is typically above 20 °C, we focused on conditions where the natural river biofilm (raised at a temperature of ca. 10 °C) would be stressed and *E. coli* growth was favored. Such conditions could become increasingly abundant in natural streams with global-warming induced temperature increases, especially in isolated pools formed during summer due to changes in discharge patterns.

To our knowledge, no prior study has examined whether altered environmental factors could influence AMR dissemination in natural river biofilms and potentially facilitate bacterial invasion under controlled laboratory conditions. A study performed by Marti et al. (2013) reported that the relative abundance of ARGs in scraped biofilm colonizing the surface of submerged stones, increased downstream of WWTP, and the associated-microbial community diversity differed from that of the upstream environment. Similarly, Reichert et al. (2021) characterized the occurrence of ARB and ARGs in river biofilms using artificial samplers and determined that biofilms contained higher abundances of ARGs in sampling sites downstream of a small WWTP. Furthermore, the biofilms also showed elevated concentrations of ARGs in comparison to the surrounding environment (surface water and sediments).

Here we considered high water temperature and flow reduction as potential promoters of bacterial invasion and increase in ARGs burden in riverine biofilms. Increased water temperature and reduced flow are relevant to typical seasonal shifts in many temperate catchments (Bourke et al., 2023; FOEN, 2021; Muelchi et al., 2021). Especially during the last decade in the Swiss Central Plateau and in the alpine southern part of Switzerland, river discharge decreases during the late spring to fall season, giving rise to rivers with interrupted flow and resulting in the creation of “refugia” (i.e., stagnant pools of various sizes and depth). Stagnant, shallow pools can easily reach high temperatures during summer days with air temperatures over 30 °C, creating conditions that are considerably different from those in a normally flowing streams. Such situations will become more frequent in the coming years due to climate alterations (Bourke et al., 2023; FOEN, 2021; Muelchi et al., 2021). Heat stress could increase the invadability of natural microbial communities and could increase the persistence of microbes adapted to higher temperatures such as fecal bacteria, thought to be important vectors for antimicrobial resistance.

The main objective of this study was to determine the effect of the above-mentioned flow and temperature alterations, on [1] the persistence of antibiotic resistance in riverine heterotrophic biofilms colonized downstream of a wastewater effluent, [2] the invasion success of the

genetically-labeled *E. coli* CM2372 as proxy of a local release of biotic pollution, and [3] the diversity and composition of the natural heterotrophic biofilm community. Due to the complexity of real river systems, we chose an experimental design that allows for control of environmental conditions. In our experiment, we exposed river-grown biofilms to an invasion of *E. coli* CM2372 within closed-circuit river water flumes under different treatments. Furthermore, we intentionally excluded the direct and continuous wastewater input and we used filtered river water to represent, at a lab-scale, the condition of fragmented or stagnant flows in heat- and drought-stressed rivers. We then tracked and quantified the abundance of *E. coli,* bacteria and resistance markers, as well as biofilm diversity and composition using quantitative PCR and amplicon sequencing over the course of two weeks. We hypothesized that warmer, stagnant water would favor the invasion success of exogenous *E. coli* invaders and increase the abundance of wastewater-associated antibiotic resistance markers.

2. Methods

2.1. Study site

The study site is located about 0.5 km downstream of the Oberglatt WWTP discharge point into the Glatt River (Fig. S1), which is a 25 km long tributary of the Thur River in northeastern Switzerland. This discharge constitutes the main source of pollution. The downstream site's river chemistry and the Oberglatt wastewater effluent (Table S0) were characterized adapting the methods of Carles and colleagues to the field (Carles et al., 2021).

2.2. Experimental design

2.2.1. Biofilm colonization

In situ colonization of biofilm was carried out over the course of two months from mid-April to mid-June 2021 within the Glatt River. Two concrete platforms covered by stainless steel chambers that allowed water flow but kept out ambient light and larger debris were installed in the river location. On each platform, sterilized glass slides were fixed onto eight artificial exposure units (Fig. S2).

2.2.2. Experimental set-up

A series of six flumes (400 × 191 × 100 mm) were assembled in a climatic chamber set to 20 °C (Fig. S3), chosen to correspond to average water temperature of the location during early summer (Federal Office for Meteorology and Climatology, 2021). Following the set-up, 16 exposure units were retrieved from the river sampling location and transported submerged in river water in sterilized plastic containers back to the laboratory. The experimental flume systems were filled with 6.5 L of filtered river water (0.45 μm membrane filters Millipore, Ireland) collected from the same site and 1.5 L was added to each 5-liter barrel connected to each flume. The exposure units were immediately installed in the covered flumes and acclimatize to experimental conditions (20 °C and continuous flow 100 mL/min) for one week (Bagra et al., 2022; Romero et al., 2019). The closed and covered recirculation systems helped prevent cross contamination and the dispersion of the genetically modified invader organisms between systems. Afterwards, varying environmental scenarios were created to determine the effect of flow (recirculating or stagnant pool formation) and the increase of the water temperature on the invasion of *E. coli* CM2372 and on the abundances of target genes present in the biofilm itself. Four of the six flumes were disconnected from the flow, and two of these were moved to a separate climate chamber set to 30 °C, corresponding to high temperatures that can be reached during the summer period in isolated small pools. This established the three treatments evaluated during this experiment: recirculating water at 20 °C (R20), stagnant water at 20 °C (S20), and stagnant water at 30 °C (S30).

2.2.3. Inoculum preparation

Escherichia coli CM2372 with *ΔlacZY* chromosomal insertion (Merlin et al., 2002) was grown overnight at 37 °C in 10 mL of Luria-Bertani (LB) broth (Sagrillo et al., 2022). One milliliter of the pre-inoculum was transferred to each one of three flasks containing 200 mL of LB broth and incubated overnight under the same conditions. The next day, each flume received an aliquot of washed *E. coli* CM2372 inoculum to achieve a final concentration of 10^7 cells/mL in the flume water. Additional details are provided in the Supplementary Information (SI).

2.3. Sample collection and physical-chemical water measurements

Each treatment consisted of two identical flumes containing exposure units with glass slides randomly sampled to obtain biological replicates (Fig. S3). To ensure sufficient biomass recovery for subsequent analysis, every replicate consisted of biofilm scraped from three microscope slides. Three biological replicates from each of the three conditions were obtained immediately after invasion at time 0 and after 1, 3, 7, and 14 days. The collected biofilm was then centrifuged and excess water was removed. The biofilm was weighed (wet biomass, mg) and the same amount (0.25 g) of biofilm stored at −20 °C for DNA extractions. Results were standardized to surface area (cm^2 glass slide surface). At each sampling date, temperature (°C), conductivity (μS/cm), pH, and dissolved oxygen (DO, mg/L), were measured (Dataset S1).

2.4. Preparation of pBELX-2 standard

The recombinant plasmid pBELX-2 (Rocha et al., 2020) was used as the qPCR standard for *ΔlacZY* strain CM2372 (Merlin et al., 2002). It was linearized by *NdeI* (Thermo Fisher Scientific, USA) before being purified using the QIAquick PCR purification kit (Qiagen, Germany) and controlled by gel electrophoresis. Additional details are in the SI.

2.5. DNA extraction and quantitative PCR (qPCR)

DNA extraction was performed using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to manufacturer instructions. Extraction blanks were used to confirm the absence of DNA contamination. The quality and quantity of extracted DNA was assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA) (Dataset S2). Extracted DNA samples were then stored at −20 °C for future analysis. DNA extracts were screened using quantitative PCR (qPCR) for the presence and abundance of seven ARGs (*sul1*, *sul2*, *ermB*, *tetW*, *tetM*, *tetB*, $bla_{CTX\text{-}M\text{-}1}$) and class 1 integron integrase gene *intI1* (Table S1), previously suggested (Berendonk et al., 2015; Gillings et al., 2014) and used (Ju et al., 2018; Lee et al., 2021) as indicator genes of anthropogenic ARG inputs. All qPCR assays, other than *ermB* and $bla_{CTX\text{-}M\text{-}1}$, were performed using Taqman chemistry on a LightCycler 480 II (Roche, Switzerland) in 10 μL final reaction volume. Primers, probes, and conditions for the target genes are given in Table S1 and detailed information regarding the qPCR procedure and standard curve efficiency are in both Table S2 and the SI (Barraud et al., 2010; Bustin et al., 2009; Chen et al., 2007; Heuer et al., 2008; Heuer and Smalla, 2007; Jung et al., 2009; Lachmayr et al., 2009; Lee et al., 2021; Walsh et al., 2011).

The abundance of *E. coli* CM2372 was determined using qPCR and the chromosomal gene tag *ΔlacZY* (Sagrillo et al., 2022). The bacterial 16S rRNA gene fragment from total biofilm was quantified using qPCR for bacterial 16S rRNA genes (Takai and Horikoshi, 2000) and then adjusted to account for *E. coli* input to distinguish it from the rest of the microbial community. The adjustment consisted of calculating the abundance of 16S rRNA Biofilm = (16S rRNA of total biofilm − 16S rRNA of *E. coli*). These values are referred to as the abundance of *biofilm-16S rRNA* genes and to the abundance of *E. coli-16S rRNA* genes in the remainder of the paper. Despite the wide use of 16S rRNA gene copies as proxy for bacterial abundance, copy number per genome may vary considerably between bacterial genomes and microbial communities. Relative abundances calculated by normalizing to 16S rRNA gene abundance therefore implicitly assume that the average copy number per genome remains constant. As total biomass and community composition did not change dramatically during

this experiment this assumption is considered reasonable. More details are provided in the SI.

2.6. Amplicon sequencing and analyses of sequence datasets

Amplicon sequencing of bacterial 16S rRNA genes was performed by Novogene using primers 341F (59-CCT AYG GGR BGC ASC AG-39) and 806R (59-GGA CTA CNN GGG TAT CTA AT-39) that target the V3-V4 region (Caporaso et al., 2011) on Illumina HiSeq technology to generate paired-end reads. The sequences were collectively analyzed using the DADA2 algorithm (Callahan et al., 2016) in QIIME2 (Bolyen et al., 2019). It resulted in 5,754,205 total high-quality reads (80 % of reads averaged ≥ Q35 scores) distributed across the 45 samples with a minimum and maximum number of reads per sample of 67,632 and 139,373, respectively. Forward and reverse reads were merged into amplicon-sequence variants (ASV) and used to build a corresponding ASV table (total 2853 ASV). More details on the bioinformatics pipeline are in the SI. All sequencing data presented in this manuscript have been submitted to the NCBI sequencing read archive under accession number PRJNA889318.

2.7. Data analyses

The dissimilarities among microbial community composition were assessed using NMDS based on a Bray-Curtis (BC) dissimilarity matrix (Legendre and Legendre, 2012). The significance of the treatments and environmental variables was tested using PERMANOVA (function *envfit* and *anosim*, Table S3). Differences in biofilm growth, *E. coli* survival, and alpha diversity metrics were analyzed using two-way ANOVA (treatments and time as fixed factors, Table 1) and one-way ANOVA (time as a fixed factor, Table S4). A combination of paired *t*-tests, Wilcoxon rank sum tests, and Kruskal-Wallis tests was applied to test differences in the abundances of the target genes, the impact of flow and temperature, and the differences in genes' abundance reduction (Tables S5, S6 and S7). All statistics and plots were produced using R software (version 4.1.1, packages: *base, car, data.table, dplyr, graphics, ggplot2, ggpubr, plyr, readxl, scales, stats, tibble, utils*, R Core Team). Additional details on statistical procedures are in the SI.

3. Results

3.1. Microcosm characterization

A Principal Component Analysis served to characterize the flumes and assess the existence of gradients in physical-chemical factors. The three treatments (R20, S20, and S30) applied to the flumes were clearly distinguished by dissolved oxygen (DO) and conductivity (58.6 % of the total variability), defining a clear separation between R20 and S30 along the first axis (PC1, r > 0.85) with S20 being intermediate (Fig. S4, Dataset S1). The tight distribution and low variability over time observed in R20 was less pronounced in S30 and not apparent in S20 (Fig. S4), as shifts in pH and DO occurred under stagnant conditions. As expected, stagnant water temperature distinguished the respective treatments, while the drift in pH is reflected in the dispersion (S20 and S30) along the second axis of the ordination (PC2, 32.7 %, Fig. S4).

3.2. Biofilm growth and E. coli survival

Overall, under recirculation flow (R20), the abundance of bacterial 16S rRNA genes on the glass slides was significantly lower than under stagnant conditions (S20 and S30) (Fig. 1, Table 1). The *biofilm 16S rRNA gene* (bacterial) abundance showed significant differences depending on treatment and sampling time (Fig. 1, Table 1). However, by the end of the experiment (day 14) the abundance of bacterial 16S rRNA genes in R20 biofilm had significantly increased and converged to values similar to those of S20 and S30 biofilms (Fig. 1, Table S4). Under stagnant conditions, the abundance of bacterial 16S rRNA genes at 20 °C had significantly increased on day 14 whereas at 30 °C it remained constant for the entire duration of the experiment (Fig. 1, Table S4).

Similarly, *E. coli* population density varied significantly depending on treatment and sampling time (Fig. 1, Table 1). The starting (day 0) *E. coli* abundance in biofilm was highest under stagnant conditions and 30 °C, and was significantly higher than at 20 °C, regardless of presence or absence of recirculation flow (Fig. 1, Table 1), which is consistent with most of the spiked *E. coli* inoculate concentration (10^7 cells/mL or, assuming 7 rRNA operons per *E. coli* chromosome, approximately 8×10^{10} 16S rRNA gene copies) initially settling onto the biofilm (Stoddard et al., 2015). At day 1, the *E. coli* abundance reached a maximum at comparable levels in both treatments with stagnant conditions (S20, S30), but remained much lower and similar to day 0 in R20. After day 1, *E. coli* abundance continuously decreased for the remainder of the experiment regardless of treatment (Fig. 1, Table S4).

3.3. Taxonomic diversity and composition of biofilm bacterial communities

In terms of relative abundance, the biofilm communities (day 0) were dominated by *Proteobacteria*, (now *Pseudomonadota*, NCBI), *Actinobacteria* (now *Actinomycetota*), *Firmicutes* (now *Bacillota*), *Chloroflexi*, and *Acidobacteria* (NCBI, 2021) (Fig. S5). During the first three days, R20 community composition significantly differed from the others (Fig. S6) although marked differences were not evident at phylum or class level (Fig. S5).

The NMDS ordination (BC distance) showed a clear and significant separation in microbial community composition according to the conditions applied (PERMANOVA, p < 0.001; Fig. 2A and Table S3), particularly between recirculation (R20) and stagnant conditions (ANOSIM p < 0.0001, R: 0.30). However, after day 3, the R20 community composition converged to the S20 and S30 community composition by the end of the experiment (Fig. 2A, day 7 and day 14). Water temperature, conductivity, and dissolved

Table 1
ANOVA two-factors and pairwise comparisons results summary (Fixed factors: Treatment and Time) for biofilm community and alpha diversity metrics across the initial time point (Day 0) and after 14 days of *E. coli* invasion. Significant differences (p < 0.5) are shown in bold.

		Treatment		Time			
		F	*p*	*F*	*p*	*F*	LM (Adj. R^2)
Community	*Biofilm-16S rRNA genes*	6.2	***0.014***	18.6	***<0.001***	2.5	0.646
	E. coli-16S rRNA genes	15.1	***<0.001***	46.9	***<0.001***	15.1	0.858
Diversity Metrics	*Shannon*	60.5	***<0.001***	70.2	***<0.001***	55.5	0.946
	Chao_1	69.3	***<0.001***	47	***<0.001***	62.9	0.947
	Faith_pd	58.6	***<0.001***	49.4	***<0.001***	42.4	0.935

Pairwise comparisons		Biofilm-16S rRNA genes (*p*)	E. coli (*p*)	Shannon (*p*)	Chao1 (*p*)	Faith_pd (*p*)
R20	*S20*	***0.02***	1	***<0.001***	***<0.001***	***<0.001***
	S30	*0.052*	***<0.001***	***<0.001***	***<0.001***	***<0.001***
	S30	1	***0.002***	1	1	0.589
S30	*R20*	*0.052*	***<0.001***	***<0.001***	***<0.001***	***<0.001***

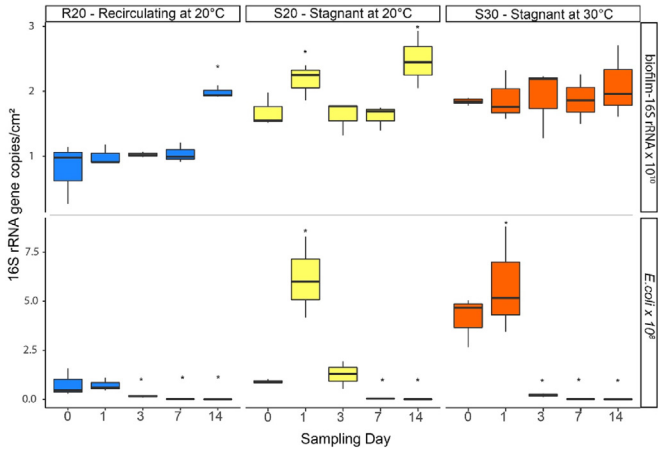

Fig. 1. qPCR-derived relative abundance of 16S rRNA gene copies/cm^2 of total bacterial 16S rRNA genes *(top)* and *E. coli* 16S rRNA genes *(bottom)* in the biofilm, collected at each sampling date from each treatment: recirculating water at 20 °C (R20), stagnant water at 20 °C (S20), and stagnant water at 30 °C (S30). The biofilm 16S rRNA abundance was calculated as = (16S rRNA of Total biofilm − 16S rRNA of *E. coli*). Asterisks denote significant differences between the initial time point (Day 0) and the indicated time points (Days 1, 3, 7, 14) for each treatment and error bars denote the standard deviation associated with the average of triplicate biofilm glass slides for each condition (ANOVA, $p < 0.05$, Table S4).

oxygen were significantly related to the differences observed in the ordination (PERMANOVA, ENVFIT test, Table S3). Significant differences were further observed between S20 and S30 in the ordination (PERMANOVA, $p < 0.001$; Fig. 2B and Table S3) and between time-points in each stagnant condition (PERMANOVA, $p < 0.05$; Fig. 2B and Table S3). In the case of stagnancy, water temperature and pH were significantly related to the differences observed in the community composition (PERMANOVA, ENVFIT test, Table S3).

The alpha diversity metrics differed among bacterial communities depending on treatment and time (Table 1). Comparisons of diversity estimators revealed that during the first three days, the biofilm bacterial community inhabiting R20 was less diverse (Shannon index), less rich (Chao1 index), and less diverse phylogenetically (Faith_pd index) than those from the stagnant conditions (Fig. 2C, Table S4). At day 3, the R20 diversity increased significantly and converged on the diversity values of S20 and S30 by the end of the experiment whereas biofilm bacteria communities inhabiting S20 and S30 showed a stable diversity across time (Fig. 2C, Table S4).

3.4. Abundance of ARGs, intl1, and ΔlacZY genes

Overall, both relative and absolute abundances of ARGs decreased over time, irrespective of water recirculation and temperature differences. The reduction in relative abundance of ARGs specifically indicated that microbial populations containing these genes were apparently less persistent than the overall biofilm community under the experimental conditions

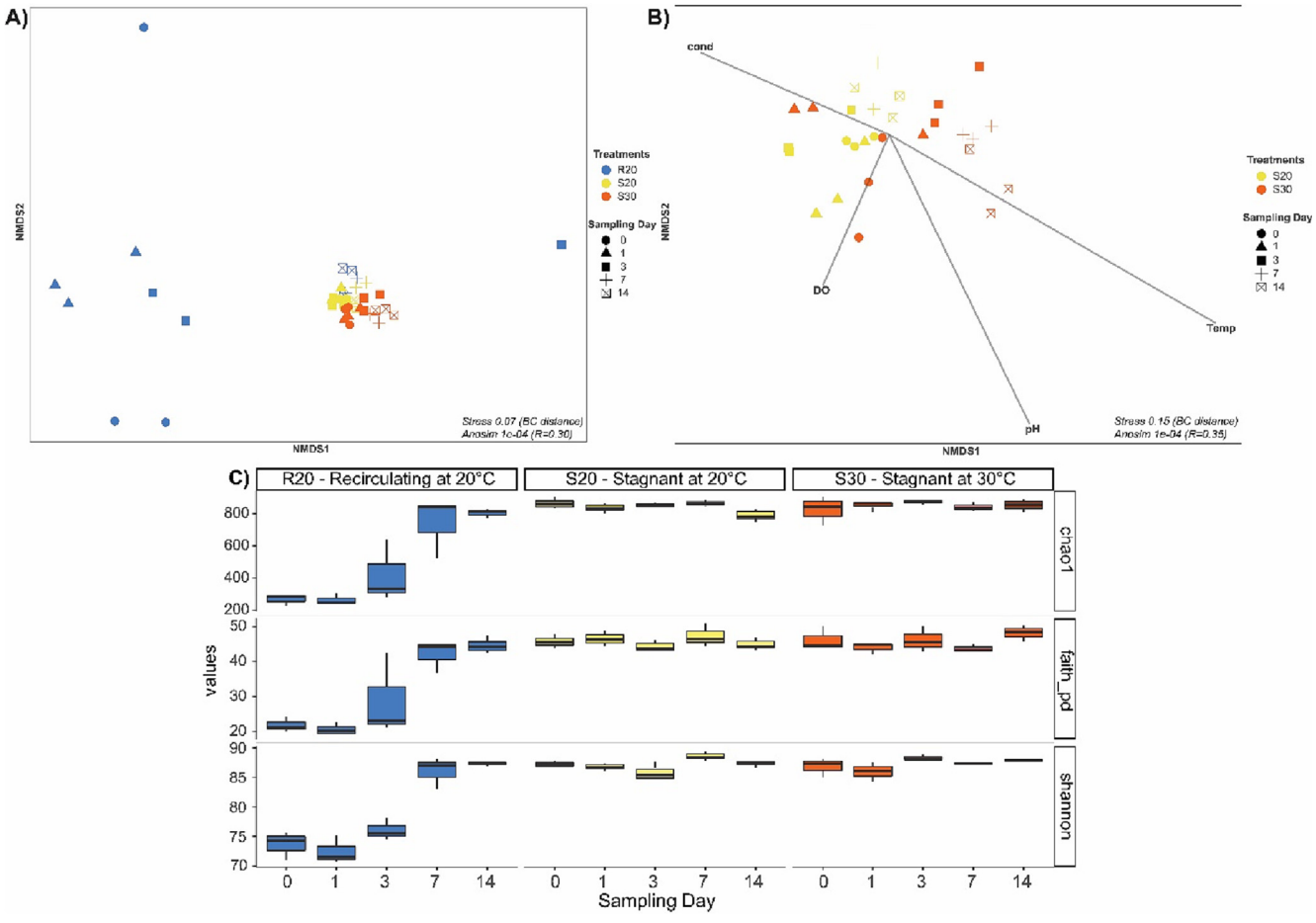

Fig. 2. A) NMDS ordination of bacterial community compositions based on Bray-Curtis distance (stress and ANOSIM test results in the figure) including all the treatments: recirculating water at 20 °C (R20), stagnant water at 20 °C (S20), and stagnant water at 30 °C (S30) and **B)** including the two stagnant treatments (S20, S30). **C)** Comparison of alpha diversity metrics (Chao1 richness; Faith_pd phylogenetic diversity; Shannon diversity index) of the biofilm bacterial communities for each condition by sampling day. Asterisks denote significant differences between the initial time (Day 0) and the indicated time points for each condition, and error bars denote the standard deviation associated with the average triplicates of each sample (ANOVA test, $p < 0.05$, Table S4).

(Fig. 3, Tables S5 and S9). Specifically, the relative and absolute abundance of genes conferring resistance to macrolides (*ermB*), sulfonamides (*sul1* and *sul2*), and tetracyclines (*tetW* and *tetM*) was similar among all conditions and had significantly decreased in R20 and S20 by the end of the experiment (Fig. 3, Wilcoxon test $p < 0.05$, Tables S5 and S9). The exception to this trend was the absolute abundance of *tetM* which experienced no significant shifts throughout the experiment. At 30 °C and stagnant conditions (S30), *tetW* and *tetM* genes remained constant and even tended to increase between day 7 and day 14. Also in this treatment, *sul2* and *intI1* showed an overall lower abundance than in the other treatments (Fig. 3A), resulting in *sul1* abundance being clearly higher than *sul2*. Both genes occurred with similar abundance in R20 and S20. Genes related to β-lactam (*blaCTXM*) and tetracycline (*tetB*) resistance were found to be below the detection limit regardless of treatment and time (data not shown).

The *ΔlacZY* gene targeting the invading *E. coli* CM2372 was detectable throughout the experiments, but it constantly decreased under continuous flow (R20), whereas it peaked in S20 and S30 treatments at day 1 before decreasing throughout the remainder of the experiment (Fig. 3, Tables S5 and S9). Peak abundances (day 1, S20 and S30) were significantly higher compared to those under continuous flow conditions at the same time (day 1, R20) (Fig. 3). By the end of the experiment its abundance had decreased by over three orders of magnitude. The potential effect of flow and temperature on the reduction of each target genes' relative and absolute abundance was assessed as R = R0-R14, which is the difference in abundance (R) between day 0 and day 14 for each gene. R values were compared between treatments (i.e. R20 against S20, S20 against S30, and R20 against S30) (Fig. 4, Fig. S7 and Table S8). For most ARGs the decrease in relative abundance was higher under flow recirculation compared to stagnancy at 20 °C (Fig. 4A, Table S6). The difference was significant for *sul1* and *intI1*

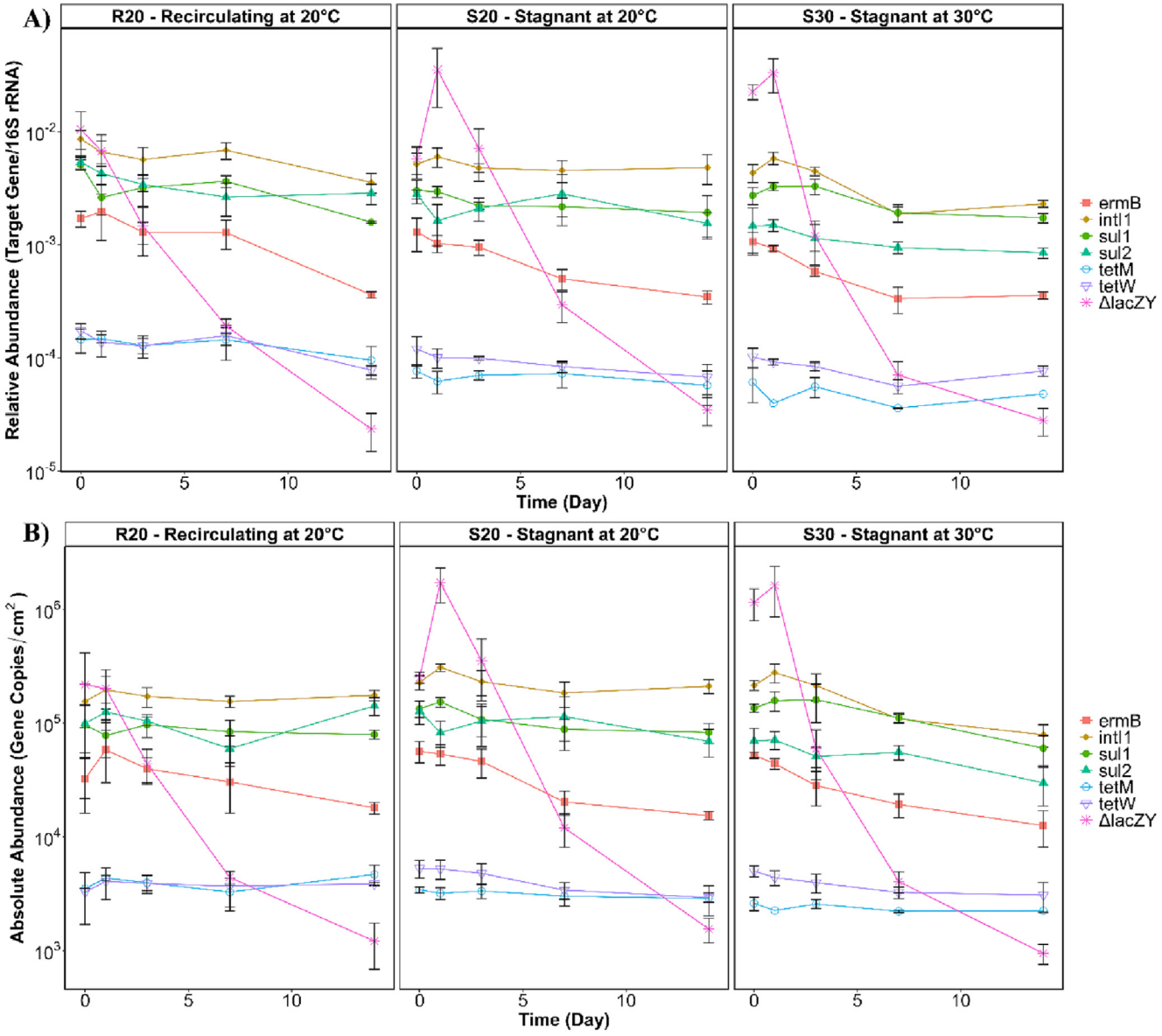

Fig. 3. A) Relative abundance of resistance indicator genes and a genomic marker of the invading *E. coli (ΔlacZY)* collected at each sampling date from each treatment: recirculating at 20 °C (R20), stagnant at 20 °C (S20), and stagnant at 30 °C (S30). The relative abundances were standardized using the biofilm-16S rRNA genes. The 16S rRNA biofilm abundance was calculated as = (16S rRNA of Total biofilm − 16S rRNA of *E. coli*). **B)** Absolute abundances for the same indicator genes and the genomic marker of the invading *E. coli (ΔlacZY)* for each treatment. The total gene copy numbers for each sample were analyzed over the sampling surface area of 118.56 cm^2 per sample. Error bars denote the standard deviation associated with the average triplicates of each sample. The significant differences between the initial time point (Day 0) and the others (Days 1, 3, 7 and 14) for each condition are summarized in Tables S5 and S9 (Wilcoxon & *t*-test, $p < 0.05$).

(Fig. 4A, Table S7). Regarding the effect of water temperature, the difference in the relative abundance reduction at 30 °C compared to 20 °C was significant only for *intI1*, for which it was larger at 30 °C (Fig. 4A, Table S7). For absolute abundances, the difference was significant for *sul1* and *intI1* at 30 °C compared to 20 °C (Fig. 4B, Table S8). The absolute abundance for both genes was higher at 30 °C. Comparing recirculation and stagnancy at 20 °C, the absolute abundance was significantly higher for *sul2*, *tetW*, and *ermB* under stagnant conditions.

4. Discussion

4.1. Biofilm development under warm and stagnant conditions

Both stagnant treatments showed a markedly different microbial community composition and increased diversity compared to the R20 condition. This indicates that the cessation of flow (all treatments were equilibrated under flow conditions before day 1 of the experiment) led to deposition and attachment of planktonic bacteria to the biofilm under stagnant conditions. The 30 °C condition was expected to represent a considerable heat stress for the biofilm community. We did not observe a decrease in abundance of biofilm bacterial 16S rRNA genes and by the end of the experiment it had increased in R20 and S20 but not in S30 (Fig. 1). However, the biofilm community composition in S30, got significantly separated from the S20 biofilm community after the first few days (Fig. 2B), indicating a potential effect of the heating water.

4.2. Dynamics of ARGs in biofilms of warm and stagnant conditions

The abundance of wastewater-associated antibiotic resistance markers in riverine biofilm decreased under all tested environmental conditions. Contrary to our initial hypothesis, neither stagnancy nor high temperature led to a proliferation (or increased persistence) of antibiotic resistance markers.

The biofilms used in this experiment were obtained from a river location in proximity to a wastewater discharge point (~500 m). The WWTP effluent is the main known and visible source of pollution in the study area (Carles et al., 2021) and the presumed origin of the ARGs and AMR markers present in the river water. The filtered river water used in the experimental flumes was collected at the same study site, ensuring that the biofilm experienced similar conditions in the flumes as in their natural habitat, but eliminating continuous exposure to WWTP effluent bacteria. Circulation flow also eliminated the continuous supply of potentially selective contaminants, e.g. antibiotics from the river water. Under these conditions, resistance markers showed signs of being slowly eliminated from the biofilm community. Both the lack of continued dispersal of resistant WWTP effluent bacteria and the loss of selective pressure (as antibiotics or other selective agents were likely degraded or adsorbed during the experiment although they were not measured) could play a role in the observed decrease. Sundqvist (2014) discussed how the reversibility of antibiotic resistance in the absence of a selective pressure depends on the fitness cost. If the fitness cost is sufficient, a population will tend to lose ARGs. However, under real-world conditions in many cases there is sufficient HGT or other mechanisms that ARGs can persist in a population for long periods of time, even if their abundance decreases (Lopatkin et al., 2017). Under natural conditions, even occasional occurrence of various selective pressures may promote persistence of resistance traits, which are frequently linked by co- or cross-resistance (Larsson and Flach, 2022). One or several of these mechanisms may explain why we observe an overall significant decrease in the abundances of ARGs within biofilm communities, but also that the genes persist (Fig. 3). Alternative explanation could be related to persister cells found deep in the biofilm layer, which are another mechanism that can support persistence of antibiotic resistance (Soares et al., 2020). Further insights into the mechanisms would require more detailed investigations than performed here.

Studies on wastewater treatment technology as promising solutions for reducing emissions of antibiotics and ARGs from wastewater (Leiva et al., 2021; Sabri et al., 2020), have not included the effect or the multiple environmental and pollutant stressors on biofilms in the receiving environments. Although the role of WWTP effluent contributing to the conveyance of AMR determinants downstream from discharge points and the relevance of the biofilm as reservoir of antibiotic resistant genes has been widely pointed out by the scientific community (Balcázar et al., 2015; Bueno et al., 2017; Rizzo et al., 2013). Applying a study design similar to ours, Romero and coworkers, examined the individual and combined effects of warming water, flow reduction, and chemical stress caused by pesticide exposure in sediment biofilms of artificial streams (Romero et al., 2019). They found that sediment biofilm, contrarily to our surface biofilm, facilitated the accumulation of toxicants, under hydrological stress and prolonged exposure to chemical pollutants (>30 days), promoting biofilm exposure and increasing toxicant abundances (Romero et al., 2019). In contrast, our lab-scale study suggested that the riverine biofilms could be remediated of antibiotic resistance markers once wastewater effluent is removed or treated to a higher standard. These potential benefits would need to be confirmed in further experiments and observational studies, but our findings highlight the potential to rapidly reduce antibiotic resistance spread in the riverine downstream biofilm compartment after environmental improvement, e.g. upgrading the wastewater treatment to release less toxicants or antibiotic resistant bacteria.

4.3. The fate of E. coli invasion in biofilms under increased temperature and flow stagnation

Regardless of changes in water temperature and flow condition, the invasion by *E. coli* was transient and not ultimately persistent. Increased temperature did not appear to improve the success of the *E. coli* invasion. The initially much higher *E. coli* abundance under stagnancy (S20 and S30) compared to recirculating flow condition (R20) was likely due the lack of shear forces and turbulence, making it easier for *E. coli* to attach to existing biofilm. This is similar to previous observations on initial biofilm formation reported by Garrett et al. (2008) where planktonic microbial cells were transported from bulk liquid to the conditioned surface either by physical forces or by bacterial motility under low turbulence conditions. On the other hand, flow can increase mass transfer and nutrient circulation, but when it passes a threshold dictated by a variety of factors, such as the interaction between a specific adhesion surface and individual microbial constituents, it can promote microbial dispersion from a biofilm community and decrease initial cell attachment (Busscher and van der Mei, 2006; Liu and Tay, 2002). Nejadnik et al. found that increasing the hydrodynamic shear stress increasingly inhibits the formation of *Staphylococcus epidermidis*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* biofilm because fewer planktonic cells were able to attach to the studied surfaces. In the R20 condition, the hydrodynamic shear force may exceed the threshold and inhibit initial *E. coli* cell attachment (Liu and Tay, 2002).

The increased attachment and reduced detachment may also have accounted for the higher abundance of biofilm bacterial 16S rRNA genes measured. It is not likely that bacterial growth would account for these differences, given the lack of significant temperature effects, and lack of further abundance of biofilm bacterial 16S rRNA genes increase in the early phases of the experiment (Fig. 1). Similarly, the differences in community composition and diversity between R20, S20, and S30 could be related to this change. Here the results suggested that stagnant conditions may have incurred both decreased microbial detachment in the absence of flow-induced shear and increased attachment of planktonic bacteria from the water.

There are a number of explanations for the ultimate failure of the *E. coli* to invade and establish itself in the biofilm. It is known, as reported by Bagra and colleagues, that the same *E. coli* strain is able to establish successfully and increase its abundance once co-exposed to chemical stressors (i.e. addition of Cu in the microcosms), most probably, due to the loss of biofilm microbial diversity observed in the co-exposure community (Bagra et al., 2022). However, they also noted that invasion success could be stochastic in nature, or additionally depended on factors not controlled by the experimental conditions. In contrast, the environmental

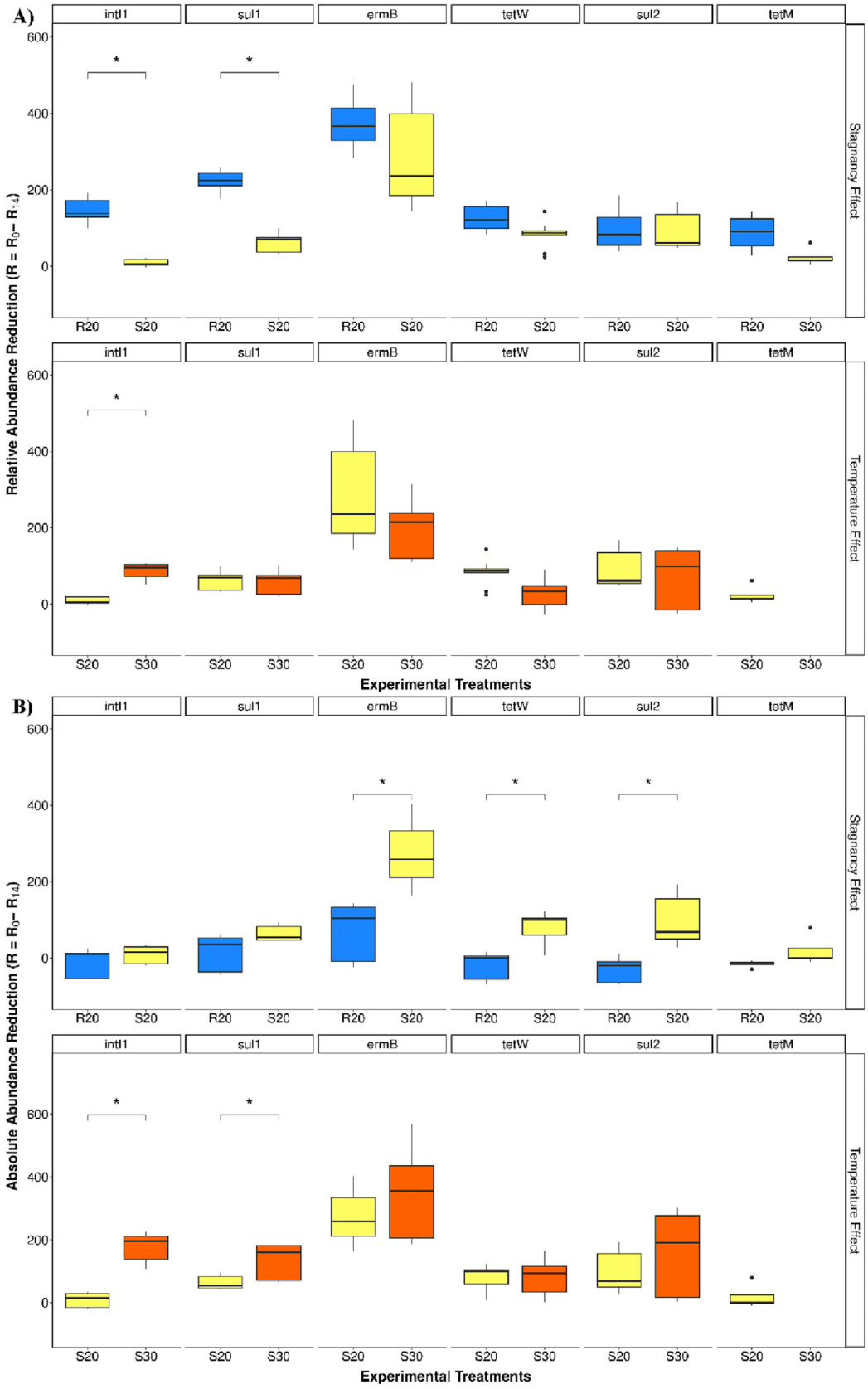

Fig. 4. A) Effect of stagnation and temperature differences on relative gene abundance changes within the sampled biofilm. **B)** Effect of stagnation and temperature differences on absolute gene abundance changes within the sampled biofilm. In the top plot of both A and B, the differences in the gene abundances (R) collected on the first (Day 0, R0) and last (Day 14, R14) sampling dates from treatments R20 (blue) and S20 (yellow) were used to evaluate the stagnancy effect, or the impact of flow recirculation (R20) vs. stagnancy (S20) at 20 °C. Difference in R collected at R0 and R14 from treatments S20 (yellow) and S30 (orange) were used to evaluate the temperature effect under stagnant conditions. In all plots, asterisks denote significant differences between the R-values of the compared treatments (Wilcoxon $p < 0.05$, Tables S7 & S9). The resistance indicator gene *tetM* is not shown in any plots for the S30 condition because there is insufficient data to calculate R.

stressors studied in our study, perhaps coupled with the absence of continuous exposure to toxicants did not trigger the establishment or proliferation of the invader nor marked changes in the biofilm diversity. Nutrient limitation and lack of competitive fitness can be a cue for individual microbial cells to release from the biofilm and sometimes the entire biofilm structure can detach in search of a different nutrient source (Hunt et al., 2004). In this study, water and nutrients were not replenished under stagnant conditions, so it is further possible that the invading *E. coli* population could not successfully access enough resources to exploit or compete with the existing biofilm community for a specific niche. However, we did not measure nutrient concentrations, so no direct evidence for nutrient limitation can be provided.

Finally, we should point out that the *E. coli* strain used in this study (CM2372) may not be indicative of the potential invasion success of all other *E. coli* strains. Nesse et al. (2014) found that not only can different *E. coli* strains exhibit varying levels of biofilm formation and have different optimal biofilm growth temperatures, but so also can different *E. coli* serotypes. As a result, future work should be done to see if the trends observed in this study are consistent for *E. coli* in general or if they are strain specific. Beyond *E. coli*, other bacteria may also have very different invasion potential that remains to be explored.

5. Conclusion

In order to improve our understanding of the environmental dimension of AMR in aquatic ecosystems we need a better understanding of biotic and abiotic factors, such as microbial invasion success of coliform bacteria or the impact of warming and stagnant waters, which could trigger the spread of AMR. Here we showed based on lab-scale results, that there was no evidence of long-lasting establishment of the exogenous *E. coli*, used as a model invader, in river-grown heterotrophic biofilms. Invaders may only be fully integrated into a natural community and experience increased persistence when ecological niches are readily available or when they have a considerable competitive advantage under the given environmental conditions.

Our study further indicated that antibiotic resistance determinants in riverine heterotrophic biofilms may decrease in abundance once wastewater effluent is removed or its load of bacteria and selective agents is reduced. This indicates there may be additional benefits for the development of new treatment strategies or the optimization of existing ones (e.g. ozonation, disinfection) to achieve efficient removal of ARB and antibiotics, as they may contribute to reducing AMR not just in surface waters but also in more persistent natural conduits of AMR, such as river biofilms.

CRediT authorship contribution statement

Giulia Gionchetta: Conceptualization, Investigation, Formal analysis, Visualization, Writing - Original Draft **Delaney Snead:** Investigation, Formal analysis, Visualization, Writing - Original Draft **Simone Semerad:** Investigation, Review and Editing **Karin Beck:** Investigation, Formal analysis, Review and Editing **Amy Pruden:** Writing - Review and Editing **Helmut Bürgmann:** Conceptualization, Funding acquisition, Supervision, Writing - Review and Editing.

Data availability

All the sequences were deposited in the NCBI (National Center for Biotechnology Information) under SRA accession (sequence read archive) number: PRJNA889318.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164658.

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