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# Cellular stress affects the fate of microbial resistance to folate inhibitors in treatment wetlands

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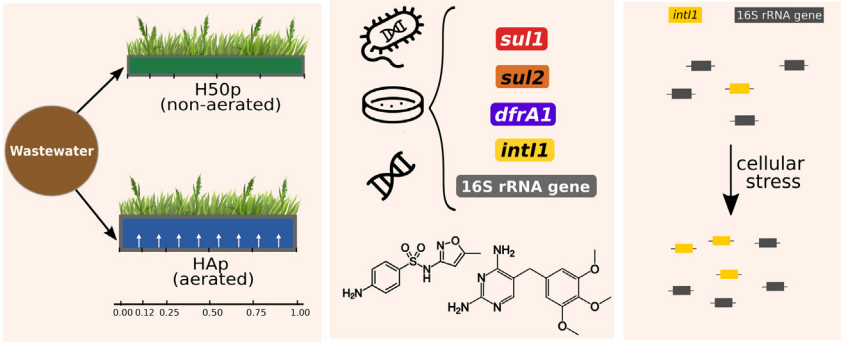
## HIGHLIGHTS

- The *sul* genes were removed by up to 4 log in aerated treatment wetland.
- Inhibitors to folate biosynthesis had no effect on prevalence of resistance genes.
- Class 1 integron diversity increased transiently in aerated treatment wetland.
- Cellular stress promotes activity of DNA recombinase, IntI1, of class 1 integron.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The environmental prevalence of antimicrobial resistance (AMR) has come into focus under the One Health concept. Wastewater treatment systems are among the significant sources of AMR in the environment. In such systems, it is uncertain to which extent antimicrobials present at sub-inhibitory concentrations constitute a selective pressure for bacterial maintenance and acquisition of antibiotic resistance (AR) genes. Here, we mapped AMR to inhibitors of folate biosynthesis in an aerated and a non-aerated horizontal subsurface flow treatment wetland receiving the same pretreated municipal wastewater. General water characteristics and the concentrations of folate inhibitors were determined to define the ambient conditions over the longitudinal axis of the two treatment wetlands. Profiling of AMR as well as class 1 integrons, a carrier of AR genes against folate inhibitors and other antimicrobials, was conducted by cultivation-dependent and -independent methods. The wetlands achieved mean reductions of AR gene copy numbers in the effluents of at least 2 log, with the aerated system performing better. The folate inhibitors had no noticeable effect on the prevalence of respective AR genes. However, there was a transient increase of AR gene copy numbers and AR gene cassette composition in class 1 integrons in the aerated wetland. The comparison of all data from both wetlands suggests that higher levels of cellular stress in the aerated system promoted the mobility of AR genes via enhancing the activity of the DNA recombinase of the class 1 integron. The findings highlight that environmental

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conditions that modulate the activity of this genetic element can be more important for the fate of associated AR genes in treatment wetlands than the ambient concentration of the respective antimicrobial agents. By extrapolation, the results suggest that cellular stress also contributes to the mobility of AR gene in other wastewater treatment systems.

## 1. Introduction

The spread of AMR is a global human health challenge (World Health Organization, 2021). Emission of treated or untreated wastewater has come under scrutiny as a substantial cause for spread of AMR via environmental sources (Berendonk et al., 2015). Although the overall abundance of AR genes decreases during wastewater treatment, there can be a relative increase of AR genes to 16S rRNA gene copy numbers (Ju et al., 2019; Majeed et al., 2021). The mechanistic reasons for the increase are still unclear. Selection at sub-inhibitory concentrations of antimicrobial compounds and enhanced mobility of AR genes at high cell densities are discussed as potential explanations (Chow et al., 2021; Manaia et al., 2018). AR gene mobility during wastewater treatment is of concern because pathogenic bacteria susceptible to antimicrobial agents may acquire clinically relevant AR genes, and pathogens that are already resistant can become multidrug resistant.

Studies addressing the mechanistic reason for the mobility of AR genes during wastewater treatment are hampered by several factors. These include the restricted ability to change the operating conditions of a wastewater treatment system for experimental purposes, and the limitation on the knowledge gained when comparing treatment systems operated differently because they do not have an identical influent source. Moreover, the microbial community composition of the influent is dynamic with various AR gene hosts and loads over time. The mixing of influent with the treatment system microbiome makes it difficult to track changes in the AR gene profiles of microbes traversing the system (Yin et al., 2019). Furthermore, AR genes are often part of mobile genetic elements present in taxonomically distinct bacteria that can respond differently to the condition of a treatment system (van Hoek et al., 2011). Although bacterial hosts of AR genes can be identified by cultivation-dependent and independent approaches (Hultman et al., 2018; Kent et al., 2020; Stalder et al., 2019), the respective methods come with bias and provide insufficient quantitative information. Therefore, it has been difficult to progress from observing correlations between AR gene prevalence and environmental conditions, such as antimicrobial concentrations, to deciphering causalities about the fate of AR genes during wastewater treatment.

Among the AR genes frequently detected in treatment systems are *sul1* and *sul2,* which confer resistance against sulfonamides such as sulfamethoxazole (SMX), and *dfrA* for resistance against trimethoprim (TMP). Their comparable high prevalence is not surprising, since these inhibitors of folate biosynthesis have been used in human medicine and animal husbandry for several decades. These compounds are typically present in the influent and effluent of treatment systems at concentrations in the ng/L up to low μg/L range (Busch et al., 2016). Measurements of in situ quantities of the *sul* genes and *dfrA1* have been used to assess the AMR status in environmental settings (Pei et al., 2006; Berendonk et al., 2015). The *sul1* gene is part of the 3′-conserved sequencesegment of the classical class 1 integron, an important vehicle for AR gene dissemination predominantly found in Gammaproteobacteria (Gillings et al., 2015; Zhang et al., 2018). Class 1 integrons are linked to transposons, potentially mobile genetic elements, which in turn can be located on conjugative plasmids. In addition to their mobility, class 1 integrons promote rapid genetic adaptation of the host by incorporating AR gene cassettes via DNA recombination (Mazel, 2006). The incorporation is catalysed by the integrase encoded by the *intI1* gene located at the 5′-conserved sequencesegment of the integron. Expression of *intI1* is not governed by specific antimicrobials but rather by the level of cellular stress. In laboratory cultures of various bacteria, including *Escherichia coli*, the expression is coupled to the SOS and stringent responses (Guerin et al., 2009; Strugeon et al., 2016), which are important regulatory schemes that enhance cell survival during physiological stress conditions. The SOS response is one of the bacterial repair systems for coping with DNA damage, and the stringent response is a reaction to nutrient limitation and other stresses. It is reasonable to assume that the SOS and stringent responses are triggered in at least some bacterial cells by the environmental conditions of wastewater treatment, e.g. via starvation and oxidative stress during aeration. As a result, *intI1* expression would be activated and the mobility of AR genes associated with class 1 integrons would be increased.

The first aim of this study was to evaluate the effect of sub-inhibitory concentrations of inhibitors of folate biosynthesis on the prevalence of *sul* and *dfrA* genes in treatment wetlands. Such systems are nature-based technologies often used for decentralised wastewater treatment (Dotro et al., 2017). The second aim was to assess whether cellular stress during wastewater treatment affects class 1 integrons in the studied wetlands. To these ends, we compared an aerated and a non-aerated horizontal subsurface flow treatment wetland receiving the same pre-treated municipal wastewater. The horizontal flow regimes allowed better capture of spatially resolved dynamics of biotic and abiotic characteristics than is possible at treatment systems with greater mixing of influent and process water. Both wetlands have been operated since 2010 with essentially steady-state removal efficiency for five-day carbonaceous biochemical oxygen demand ($CBOD_5$), inorganic nitrogen species, and *E. coli* (Headley et al., 2013; Nivala et al., 2013b; Kahl et al., 2017). The decrease in $CBOD_5$ and the increase in dissolved oxygen concentration have been about 5–10 times greater in the aerated wetland as compared to the non-aerated wetland, making it likely that in the former system bacteria not adapted to the habitat experience more starvation and oxidative stress.

## 2. Materials and methods

### 2.1. Horizontal subsurface flow constructed wetlands

The two pilot-scale horizontal subsurface-flow treatment wetlands investigated are located at the Langenreichenbach Ecotechnology Research Facility 42 km north-east of Leipzig, Germany (51.50613 N, 12.89758E). At the Research Facility, approximately 16.5 $m^3\ d^{-1}$ of raw wastewater from the surrounding rural-residential villages is primary treated in a three-chamber septic tank and from there distributed in parallel to the wetlands as only source of water other than precipitation (Nivala et al., 2013a). The water coming from the septic tank and entering the wetland inlets is referred to as fractional length 0.00. The principal operational difference between the systems is that one is aerated via a tubing system installed at the bottom perpendicular to the flow direction, while the other system is not aerated. The aerated system is named HAp (for Horizontal Aerated and planted), and the non-aerated wetland is named H50p (for Horizontal 50 cm depth and planted). Both systems have a surface area of 5.6 $m^2$ and are planted with common reed (*Phragmites australis*) (Fig. 1a, Fig. S1). The depths of HAp and H50p are 100 cm and 50 cm, respectively. Details on the operation of the two wetlands can be found in Table S1a and in the literature (Nivala et al., 2013a).

### 2.2. Sampling and analysis of conventional wastewater characteristics

There were 11 sampling campaigns during the vegetation period of four years (May 2014–October 2017). For reasons of workload, the full set of analytes was typically recorded over two or more successional sampling days. Counting of *E. coli* colony forming units (CFU) was done only for 4 sampling campaigns since the results were similar to most probable number (MPN) counts (see Sections 2.5 and 3.5). Samples were taken from the septic tank as the common inflow, the effluents, and from inside the treatment wetlands at 0.12, 0.25, 0.50, 0.75 of the fractional length and 0.50 of the fractional depth via permanently installed tubes. The wastewater

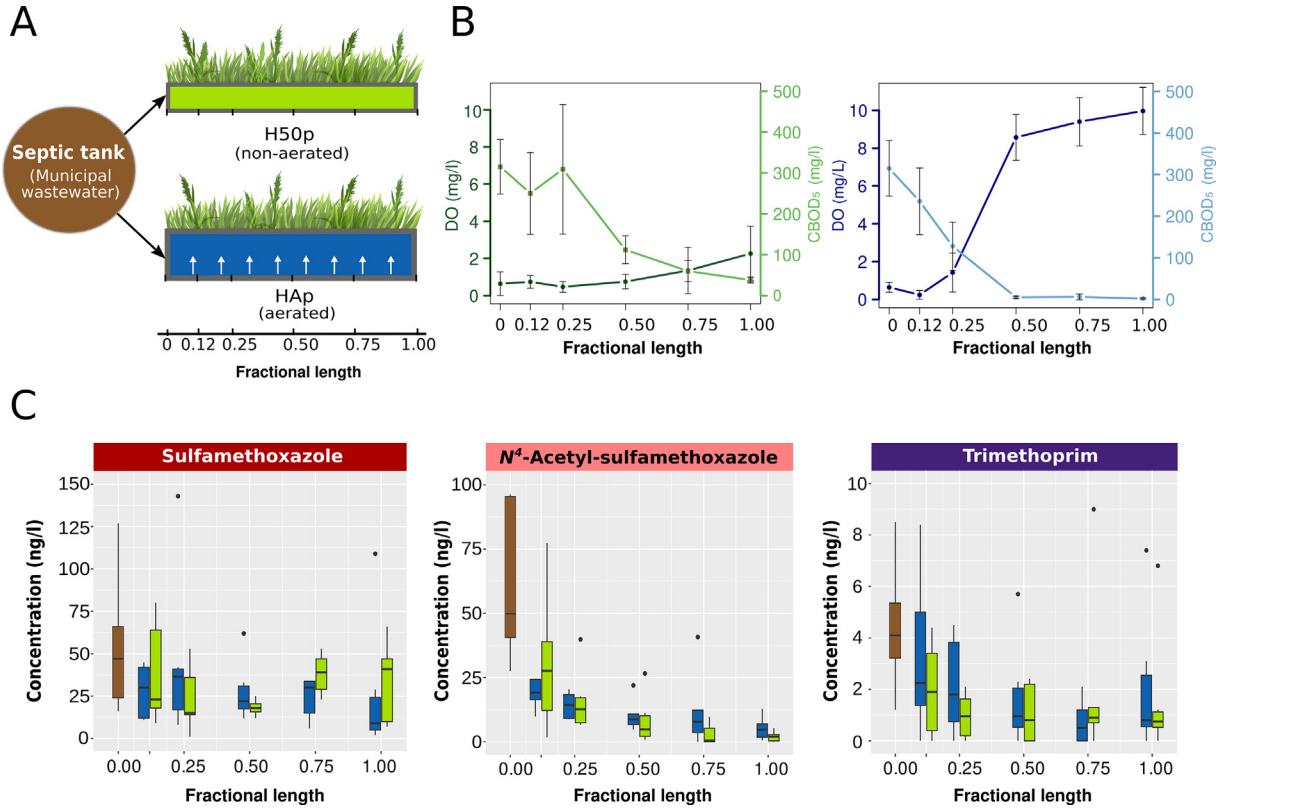


**Fig. 1.** Chemical water characteristics along the flow paths of the treatment wetlands HAp and H50p. A) Schematic representation of the wetlands and the sampling positions as fractional lengths of the wetlands. White arrows in HAp indicate aeration. B) Dissolved oxygen (DO, dark colours) and carbonaceous oxygen demand ($CBOD_5$, light colours) along the flow path for HAp (blue) and H50p (green) ($n$ = 9 time points). C) Sulfamethoxazole (left), $N^4$-acetylsulfamethoxazole (middle) and trimethoprim (right) concentrations in the influent (brown), HAp (blue) and H50p (green) ($n$ = 6 time points).

characteristics temperature, pH, nitrogen species, and dissolved oxygen were determined on site according to standard protocols (Kahl et al., 2017). The remainder of the sample volume was transported to the laboratory under refrigeration. Upon arrival, a portion of the samples was immediately used for bacterial culturing on agar plates and another portion for DNA extraction.

### 2.3. HPLC-MS-MS analysis of the selected antibiotics in wastewater

Wastewater samples (250 mL) were filtered (0.45 mesh, glass fibre filter, GE Healthcare, Buckinghamshire, UK) and extracted by solid phase extraction (SPE) using Oasis HLB (200 mg, Waters, Milford, USA). Before applying the sample, the SPE sorbent was conditioned with methanol (Biosolve, Dieuze, France) and Milli-Q water. After washing with 2 mL Milli-Q water, the sorbent was dried for 30 min under a gentle stream of nitrogen gas. The analytes were eluted with 10 mL methanol and concentrated to 0.5 mL (TurboVap II, Biotage, SWE). Addition of 0.5 mL water with 5 mM ammonium acetate and 0.1 % formic acid (HPLC solvent A) gave the final volume of 1 mL. An aliquot of 5 μL was injected for High Performance Liquid Chromatography—tandem Mass Spectrometry (HPLC-MS-MS) analysis.

Samples for suspended solids were collected from H50p and HAp at 25 cm and 50 cm depth, respectively. Samples were filtered (45 μm pore diameter), and the solid material was washed with MilliQ-water and air-dried. Between 0.006 g and 2.2 g of sludge (dry weight) were extracted twice with 10 mL methanol in a sonication bath for 15 min. After centrifugation at 5000 rpm for 20 min (Centrifuge 5804, Eppendorf, Germany), the methanol was evaporated to a final extract volume of 1 mL, which was used for HPLC-MS-MS analysis.

The analysis was carried out with an Agilent 1260 HPLC instrument (Agilent Technologies, Waldbronn, Germany) coupled to a triple stage quadrupole mass spectrometer ("QTrap 5500", SCIEX, Darmstadt, Germany). Chromatographic separation was performed with an "Ascentis Express C18" column (10 cm × 3 mm i.d. and 2.7 μm particle size, Supelco, Seelze, Germany). Solvent A and methanol with 5 mM ammonium acetate and 0.1 % formic acid (solvent B) were used to elute the antimicrobials at a flow rate of 300 μL $min^{-1}$ with the following linear gradient: zero to 1 min 95 % solvent A, 1 to 15 min 10 % solvent A, from 20 to 25 min 95 % solvent A. The temperature of the column oven was maintained at 30 °C. Electrospray ionization was operated in positive mode with 5.5 kV spray voltage. Mass analysis in multiple reaction monitoring mode was used with the analyte-specific ion transitions (Table S2). Calibration curves were generated for quantification of the analytes with 5 concentrations measured twice before and after each sample batch. Duplicates of each sample were measured randomly distributed in an analysis batch. Instrument blanks (methanol and tap water injections) were included multiple times in the sample batches. SPE enrichment recoveries ranged from 89 % to 111 %. Instrumental limits of detection and methodological limit of quantification ($LOD_i$, $LOQ_m$) as well as analytical precision are given in Table S2.

### 2.4. Amplicon sequencing of the 16S rRNA gene

For DNA extraction, samples were centrifuged at 13,000 rpm for 15 min. The sample volume (1.5 to 60 mL) was dependent on the origin of the sample in the flow path through the wetlands. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). DNA concentrations were quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) or Qubit (Thermo Fisher Scientific).

PCR amplicons of variable region V3 of the 16S rRNA gene were generated essentially according to an established protocol (Vollmers et al., 2017) using forward primer 341bf (Herlemann et al., 2011) and reverse primer uni518r (Muyzer et al., 1992) (Table S3), with the modifications that amplicons were created using the extracted DNA directly as template without prior multiple displacement amplification. Universal Illumina adapters were already included as primer overhangs. Sequencing library preparation and Paired-End sequencing (2 × 150 bp) were carried out using an Illumina Nextseq-System (Illumina, San Diego, CA, USA). Data analysis was done using R (R Core Team, 2020) with the packages DADA2 (Callahan et al., 2016), phyloseq (McMurdie and Holmes, 2013), Biostrings (Pagès et al., 2019) and ggplot2 (Alboukadel, 2018). Phylogenetic assignments were computed using the SILVA database, release 132 (Quast et al., 2012). Sequence reads are archived at GenBank under accession number PRJNA773502.

### 2.5. Cultivation-dependent quantification of Escherichia coli

Culturable *E. coli* were counted as MPN, adopting the Colilert®-18 Quanti-Tray method (IDEXX Laboratories, USA), and by CFU counting on Endo Agar (Sigma-Aldrich).

### 2.6. Quantitative PCR enumerations of AR genes, intI1, and phylogenetic marker genes

DNA was extracted in the same manner as for Illumina sequencing. The qPCR analysis was carried out as described before with primers for *sul1*, *sul2*, *dfrA1*, *intI1*, 16S rRNA gene, and the *uidA* gene for total *E. coli* (Table S3). The average efficiency values were 84.5 % for *sul1,* 94.1 % for *sul2,* 90 % for *dfrA1*, 91.3 % for *intI1*, 90 % for the 16S rRNA gene, and 95 % for *uidA*. We refer to 'fold change' as the change in copy number abundance of an AR genes, 16S rRNA gene, or *intI1*, X for short, at the given location compared with that in the influent: fold change = $(X_{\text{fractional length}}\text{-}X_{\text{inlet}}) / X_{\text{inlet}}$. qPCR data are available at figshare under https://doi.org/10.6084/m9.figshare.16862008.v1.

### 2.7. Isolation and characterization of SMX and TMP resistant isolates

Isolation of resistant bacteria present in water samples was carried out on Mueller-Hinton agar (Carl Roth), Endo Agar (Sigma-Aldrich), Aeromonas Isolation Agar without ampicillin added (Sigma-Aldrich) and Pseudomonas Isolation Agar (Sigma-Aldrich) as described elsewhere (Czekalski et al., 2012). Enumerations were made in duplicate with and without the addition of SMX (64 mg/L) or TMP (8 mg/L) to the media. SMX- and TMP-resistant isolates were selected from the various agar plates, sub-cultured on their respective isolation media, and stored in 15 % (*w/v*) glycerol at −80 °C.

For sequencing of the 16S rRNA gene and identification of AMR determinants in cultured SMX- or TMP-resistant bacteria, genomic DNA was extracted by the microwave boiling method (Orsini and Romano-Spica, 2001). Extracted DNA was stored at −20 °C until used. The 16S rRNA gene was PCR-amplified, and the presence of *sul1, sul2*, *dfrA1*, *intI1*, and gene cassettes was PCR-tested for using REDTaq® ReadyMix™ PCR Reaction Mix and the primers listed in Table S3. PCR amplicons of 16S rRNA gene and gene cassettes were purified by using MinElute PCR purification Kit (Qiagen, Valencia, CA, USA) and sequenced by the Sanger method (Macrogen, Netherlands). Phylogenetic trees of the 16S rRNA gene were built and drawn in R using the packages 'ggtree' and 'tidytree' (Yu et al., 2017), 'ggplot2' (Alboukadel, 2018), 'phangorn' (Schliep et al., 2017), 'ape' (Paradis and Schliep, 2019), seqinr (Charif and Lobry, 2007) and 'treeio' (Wang et al., 2020). The Maximum Likelihood method with Jukes-Cantor model were chosen and a bootstrapping of 100. For alignment, the online tool SILVA Incremental Aligner (SINA) on the SILVA database was used. The classification of cassettes was done according to amplicon sizes and Sanger sequencing.

### 2.8. Statistics

Statistics were carried out in R studio using the package ggpubr (Alboukadel, 2018).

## 3. Results and discussion

### 3.1. Water chemistry of the treatment wetlands

In this and the following section, we describe the abiotic and biotic conditions along the longitudinal axes of the two treatment wetlands. A detailed characterization of the environmental conditions is important to gain mechanistic insights into the mobility of AR genes in the systems. During the four-year experimental period, the treatment performance of the systems was stable according to weekly monitoring of conventional water parameters in the influents and effluents as well as measurements recorded during 11 sampling campaigns from four fixed positions each within HAp and H50p. Fig. 1b shows that between 0.25 and 0.50 of the fractional length of HAp, the dissolved oxygen conditions changed from anoxic or hypoxic to oxygen (hyper)saturation. Concomitantly, concentrations of $CBOD_5$ and $NH_4^+$-N were attenuated to < 5.0 mg/L and < 0.2 mg/L, respectively, during the first half of HAp. The concentrations of $CBOD_5$, dissolved oxygen, and ammonium in the effluent of HAp were similar to that of a standard activated sludge treatment system. $NO_3^-$-N was below the limit of detection (LOD) of 0.07 mg/L in the influent and increased to a concentration of about 45 mg/L in the second half of the HAp wetland (Fig. S2). $NO_2^-$-N was almost always below the LOD of 0.6 μg/L. Exceptions were nine samples from fractional length 0.25 and 0.50 with $NO_2^-$-N concentrations between 0.1 and 0.8 mg/L. High concentrations of dissolved oxygen and nitrate in the second half of HAp show that the aqueous phase was electron-donor limited for microbial respiration. In contrast, dissolved oxygen remained low over the length of the non-aerated wetland H50p, reaching 2.3 ± 1.5 mg /L in the effluent, and $CBOD_5$ decreased gradually to 37 ± 7 mg/L (Fig. 1b). The concentration of $NH_4^+$ —N was only attenuated to 61.4 ± 11.7 mg/L (15 % decrease compared to the influent), and $NO_3^-$-N was below the LOD in the effluent of H50p. The pH was between 7.2 and 7.5 in both systems, and the average water temperature was 16 ± 4 °C. The comparison of the concentration dynamics of $CBOD_5$ and $NH_4^+$-N in both wetlands show that aeration occurred over the entire flow path of HAp. Therefore, the anoxic and hypoxic condition in the first part of the system was due to microbial aerobic respiration rather than hypothetical clogging of the air distribution system.

SMX, $N^4$-acetyl-SMX, sulfadiazine, sulfamethazine, and TMP were quantified by LC-MS-MS in samples taken along the flow paths (Fig. 1c; data not shown for sulfadiazine and sulfamethazine). $N^4$-acetyl-SMX is the main metabolite of SMX transformation by warm-blooded animals and humans. It has no antimicrobial activity but can be transformed back to its parent compound in microbial communities (Gobel et al., 2007). In the influent and throughout the flow paths, mean concentrations of SMX and $N^4$-acetyl-SMX were < 60 ng/L, and < 5 ng/L of sulfadiazine, sulfamethazine and TMP, all of which are in the low-to-middle concentration range for wastewater-impacted surface waters in Germany (Busch et al., 2016). The concentrations of sulfonamides and TMP found here are at least five orders of magnitude lower than the reported Minimal Inhibitory Concentrations (MIC) (Czekalski et al., 2012). Mean concentrations of the measured compounds were very similar in both wetlands at sampling sites with the same fractional length. Thus, a hypothetical effect of sub-inhibitory concentrations on the prevalence of AR genes conferring resistance to sulfonamides or TMP would have been essentially the same in both wetlands.

### 3.2. Structures of the bacterial communities

The microbial community profiles were assessed by Illumina sequencing of 16S rRNA gene amplicons. Illumina data was generated from 66 samples taken along the flow paths. A total of 5,143,615 high-quality sequences

were generated (on average 7.9 × $10^4$ sequences per sample), with Proteobacteria, Firmicutes, and Bacteroidetes being the dominant phylotypes (Fig. S3). Richness and alpha-diversity were similar for all but one sample based on values for Amplicon Sequence Variants (ASV = 2749,2 ± 1404,1, Table S4) and Shannon index (H′ = 7,38 ± 0,70, Table S5). These values were within the range previously reported for treatment wetlands receiving municipal wastewater (Fu et al., 2019; Guan et al., 2015).

The beta-diversity of the microbial communities along the flow paths were visualized by principal coordinate analysis (PCoA) of the Bray-Curtis dissimilarity index (Fig. 2a). The community profiles from the non-aerated wetland H50p were more similar to those from the influent than to the communities in samples from the aerated wetland HAp. The profiles of HAp clustered into two groups, one with the communities from the first half of the wetland and another one with samples from the second half. The

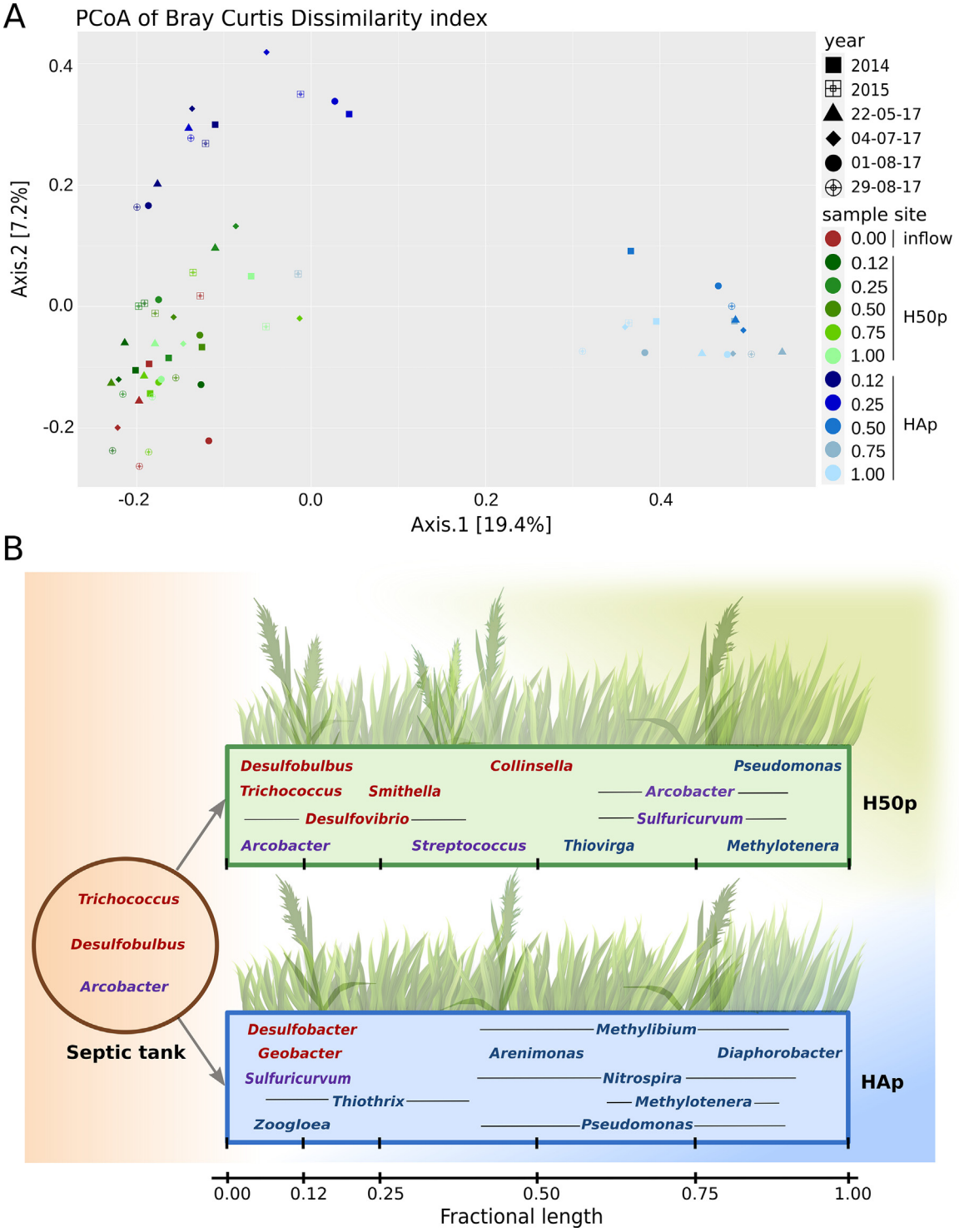


**Fig. 2.** Microbial community structures in the treatment wetlands H50p and HAp. A) Principal coordinate analysis of Bray-Curtis dissimilarity index of 16S rRNA gene amplicons ($n$ = 65). B) Representative selections of the top most abundant genera at the different sampling points of the two wetlands. Names of taxa growing anaerobically are given in red colour, facultative anaerobes in purple, and taxa typically growing aerobically in blue.

cluster in the first half was closer to the communities from the influent and H50p, while the communities from the second half of HAp were markedly different from the others.

The taxonomic compositions of the microbial communities fitted to the dynamics of the conventional water parameters along the flow paths of H50p and HAp (Fig. 2b; Table S6, available at https://doi.org/10.6084/m9.figshare.17004919). The community in the influent was dominated by anaerobic bacteria typically found in the gut of warm-blooded animals and humans, such as members of the Lactobacillales, and by microbes thriving on the reduction of inorganic sulfur species, such as *Desulfarculaceae*, *Desulfobacteraceae* and *Desulfobulbaceae*. In H50p, the predominant taxa were strictly anaerobic bacteria, many of which were also found in the influent. In contrast, the microbial community throughout HAp was mostly comprised of bacteria described as growing aerobically or by denitrification. The relative abundance of their 16S rRNA genes in the first half of HAp together with the transformation of $NH_4^+$-N into $NO_3^-$-N showed that oxygen was an important electron acceptor in this wetland section. The community in the second half of HAp consisted mostly of microbes frequently found in association with plants, such as *Rhizobiaceae* and *Burkholderiaceae*.

### 3.3. Abundance of AMR indicators

The abundance of the 16S rRNA gene and of the AMR indicator genes *intI1*, *sul1, sul2*, and *dfrA1* was recorded in the treatment wetlands by qPCR. Initial screening with standard PCR on 55 water samples showed that *sul1* and *sul2* but not *sul3* were always detectable. Among *dfrA* alleles commonly found in Europe, *dfrA1* was detected most frequently in our PCR screening, while *dfrA5*, *7*, *12* and *17* were not found in all tested samples. Copy numbers of *intI1*, *sul1*, *sul2*, *dfrA1* and the 16S rRNA gene were then enumerated by qPCR in the influent, effluent, and internal samples from both treatment wetlands. To first asses overall treatment performance, the absolute abundance of the AMR indicators in the influent were compared against those in the effluent. HAp and H50p achieved mean reductions of AR gene copy numbers of at least ~2 log (Fig. S4), which is in a similar range to other treatment technologies such as activated sludge-based systems and membrane reactors (Munir et al., 2011) and better than reported for treatment wetlands operated for a shorter period of time (Chen et al., 2016; Yi et al., 2017; Liu et al., 2019). Notably, reduction in mean copy numbers of *intI1* and *sul1* was about 3 log and 2 log units better in HAp than in H50p. This difference was at least one log unit greater than the difference in mean copy numbers of *dfrA1* in the two effluents, revealing different fates of the AMR indicators in the two systems.

Fold changes of absolute abundances at sampling points in comparison to the influent were calculated to normalize the data for possible temporal fluctuations in the incoming wastewater. Fold changes differed among treated wetland types and, in the case of HAp, also among AMR indicator genes (Fig. 3). All mean fold changes were < 0.5 along the flow path of H50p, i.e., there was no significant increase in gene abundance in the system. In contrast, in the first half of HAp, the mean fold changes of *intI1* and both *sul* genes were > 1. These fold changes were statistically significant (Wilcoxon test, $p$-values < 0.05 and < 0.01). Quantification of *intI1* was done in all samples ($n = 11$) with primers targeting both clinical and environmental alleles (Mazel et al., 2000). Fold changes of *intI1* in the first portion of HAp were slightly higher with primers targeting the classical clinical allele (Gillings et al., 2015; $n = 6$; Fig. S5). Onwards from 0.50 of the fractional length of HAp, the fold changes of these three genes were < 0. The fold changes of *dfrA1* were < 0 throughout the flow path of HAp. Likewise, the mean fold changes of the 16S rRNA gene were < 0 at all sampling sites of both systems.

The difference in fold change patterns of *intI1* and the *sul* genes at essentially the same sulfonamide concentrations in the two treatment wetlands argue against an effect of the antimicrobials on the prevalence of the genes. It is also unlikely that bacterial density played a role in the transient increase in HAp, as 16S rRNA copy numbers decreased similarly in both wetlands from the influent to the last sampling site. Previous studies that found correlations between sub-inhibitory concentrations of sulfonamides and the abundance of respective AR genes in the environment did not fully consider that antimicrobials are typically emitted together with AR bacteria and their genes. We note that such correlations can be a consequence of the extent of in situ mixing of microbial communities with different AR gene profiles. Here, the horizontal flow regimes of the treatment wetlands allowed to show that the transient increase in prevalence of *intI1* and the *sul* genes in the aerated system occurred while the concentrations of sulfonamides decreased. Currently, we can only speculate that the prevalence increase was due to enhanced mobility of the gene-carrying elements, such as conjugative plasmids.

### 3.4. Prevalence of AR genes and diversity of class 1 integrons in isolates from the wetlands

Next, we isolated and characterized SMX- and TMP-resistant strains to explore the distribution pattern and dynamics of class 1 integrons and the AR genes in culturable members of the bacterial community in the influent and along the flow path of HAp and H50p. We reasoned that the ability to check for co-localization of the AR genes in isolates outweighs the limitation of this approach due to isolation bias. For an initial screening, Müller-Hinton Agar was used as general-purpose medium. Based on Sanger sequencing of the 16S rRNA gene of 24 isolates, most SMX and TMP resistant strains belonged to either the family *Enterobacteriaceae* (12 isolates) or the genera *Aeromonas* (7 isolates) and *Pseudomonas* (4 isolates). To focus our investigations on these phylogenetic groups, further isolation was then carried out with the selective media Aeromonas Isolation Agar, Pseudomonas Isolation Agar, and Endo Agar, ultimately yielding 1624 strains (Tables S9-S10). Strain identity was revealed by Sanger sequencing of the 16S rRNA gene or PCR-based testing for the *uidA* gene of *E. coli*. The strains were then PCR-screened for *intI1*, *sul1, sul2, sul3,* and *dfrA* alleles. Approximately 90 % of the SMX- and/or TMP- resistant isolates recovered from the common influent carried at least one of these genes. The taxonomic distributions of *sul1* and *intI1* hosts overlapped, with *Aeromonas* spp. as the most abundant group followed by *Enterobacteriaceae* and *Pseudomonas* spp. (Fig. S6). The three phylotypes affiliate to the Gammaproteobacteria, and thus the result fits with the outcome of a survey of the prevalence of class 1 integrons in sequenced bacterial genomes (Zhang et al., 2018). The detection frequency of *intI1*, *sul1* and *sul2,* and *dfrA* remained about the same in all isolates recovered from along the flow path of H50p. In contrast, the frequency of *sul* and *dfrA* in the resistant strains isolated from the HAp system decreased substantially along the flow path, being almost zero in the isolates from the effluent. The larger decrease in gene prevalence among the isolates from the second half of HAp fitted to the qPCR-based enumerations. The change among the isolates from HAp is partially due to a shift in the bacterial community to species that are intrinsically resistant to the antimicrobials, like *Pseudomonas* spp. or *Enterococcus* spp. Moreover, of the resistant isolates belonging to *Aeromonas* or *Enterobacteriaceae*, more strains from the influent than from the effluent of HAp carried the AR genes. Some isolates carried *intI1* but not *sul1*, while with others it was vice versa. This absence of co-occurrence was mostly the case with isolates recovered on Pseudomonas Isolation Agar with SMX as selective marker in the medium, e.g., all strains isolated from the influent harboured *sul1*, while only 60 % of the strains carried *intI1*. This finding mirrors the qPCR results where the abundances of *sul1* and *intI1* were similar yet not identical. The genes *sul2* and *drfA1* co-localized with *intI1* in only 6 % and 9 % of the isolates, respectively, most of which were members of the *Enterobacteriaceae*.

To investigated if the environmental dynamics in the wetlands had an effect on class 1 integrons, we characterized the variable regions of the integrons of 219 SMX resistant isolates. The regions were PCR-amplified with primers binding to the 5′- and 3′- conserved sequencesegment of the integron. An isolate is regarded as carrying several class 1 integron copies with various gene cassettes when multiple bands are detected during gel electrophoresis of the PCR product. We refer to those isolates as polycarriers. Isolates from the first half of HAp had higher integron cassette

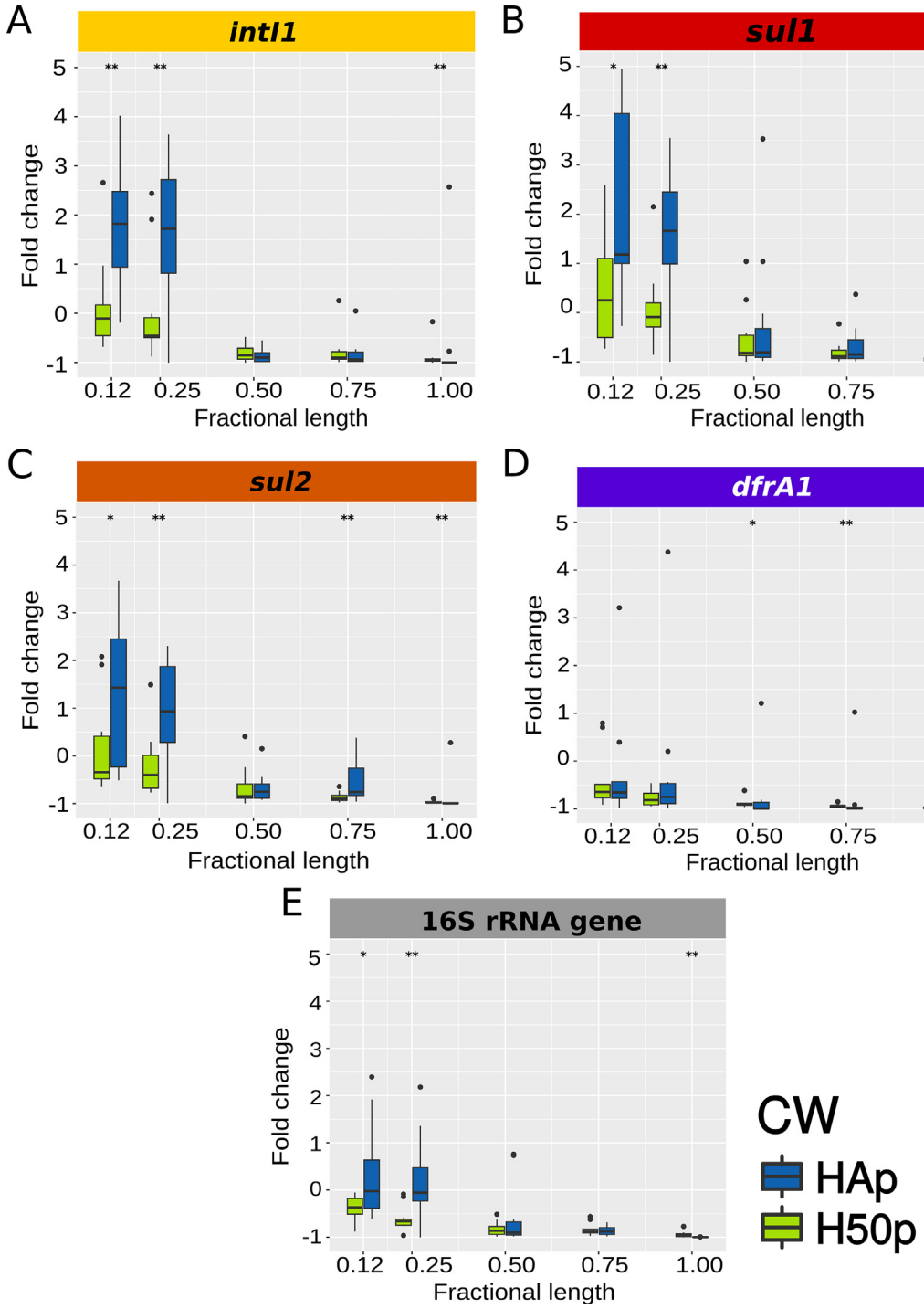


**Fig. 3.** Fold change of gene abundances along the flow path of the treatment wetlands. Computation of fold changes based on qPCR enumerations with samples ($n$ = 11 time points) from the aerated treatment wetland HAp (blue boxes) and non-aerated treatment wetland H50p (green box). Fold change = ($Abundance_{fractional\ length}$ - $Abundance_{influent}$) / $Abundance_{influent}$. A) *intI1*; B) *sul1*; C) *sul2*; D) *dfrA1*; E) 16S rRNA gene. Boxplots represent the median and the interquartile range of the fold change of gene abundances. Statistical significance of fold changes was calculated with Wilcoxon test (*$p < 0.05$; **$p < 0.01$).

diversity than those from H50p (Fig. 4). Sanger sequencing of representative PCR products showed that the AR genes in the variable regions were *aadA1, aadA2*, *aadA4* (conferring streptomycin resistance), *catB3* (chloramphenicol resistance), *dfrA1*, and *ereA* (erythromycin resistance) in various combinations (Table S9). The increase in polycarrier diversity in isolates within narrow taxonomic ranges reveals that rearrangement of AR gene cassettes had occurred. Since all isolates were cultured under the same conditions in the laboratory, the rearrangements must have taken place in the wetlands, particularly in HAp. Consequently, since the expression and thus the activity of the integrase that mediates gene cassettes rearrangement is enhanced when the SOS and/or stringent responses are activated (Guerin et al., 2009; Strugeon et al., 2016), the higher polycarrier diversity is evidence of increased cellular stress levels in the hosts of class 1 integrons in the wetlands. The decline in polycarrier diversity in the second half of HAp may be due to the substantial replacement of the influent community with microbes already present in the wetland.

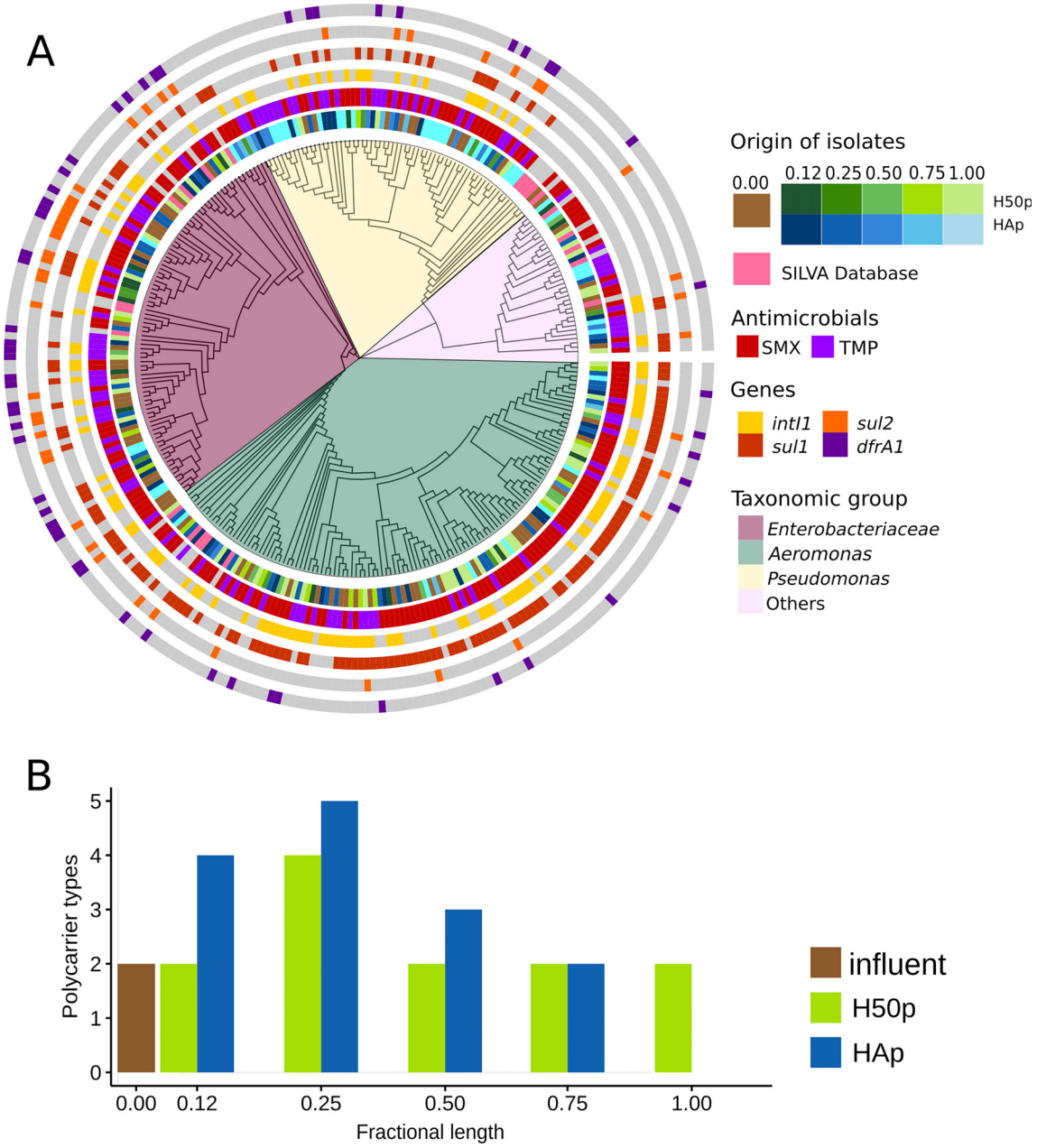


**Fig. 4.** Presence of AR genes and class 1 integrons in isolated strains. A) The phylogenetic relation and AR gene content of 290 SMX and TMP resistant isolates from the septic tank and from the sampling points of the treatment wetlands HAp and H50p are depicted. Sequences of reference strains were obtained from the SILVA database. The rings indicate from inside to outside: isolation source, antimicrobial used for selection, presence of *intI1*, *sul1*, *sul2* and *dfrA1*. B) Number of different types of class 1 polycarriers isolated from the influent (brown) and along the flow path of HAp (blue) and H50p (green).

### *3.5. Viability of E. coli in the treatment wetlands*

To obtain further evidence that the ambient conditions in the first part of the aerated wetland HAp caused cellular stress in hosts of class 1 integrons, the viability of *E. coli* in this system was compared with that in the non-aerated H50p. The rationale for this was as follows. First, *E. coli* accounted for a sizeable portion (about 20 %) of isolates from the wetlands that harboured at least one class 1 integron copy. Second, *E. coli* likely experienced in situ starvation for glucose, its preferred catabolic substrates, and other nutrients, which can trigger the SOS and stringent responses, especially under aerobic conditions (Taddei et al., 1995; Chang et al., 2002). Furthermore, the viability of starved *E. coli* declines more rapidly under aerobic than under anaerobic conditions due to oxidative damage of macromolecules including DNA (Dukan and Nyström, 1999). Third, there are analytical means to accurately assess the viability of *E. coli* in a complex community in a large habitat. We chose to compare viability counts determined by cultivation-dependent methods with qPCR-based quantification of the *uidA* gene of *E. coli* as cultivation-independent surrogate for measuring total cell counts.

The mean abundance of culturable *E. coli* was approximately $1 \times 10^6$ cells/100 mL in the influent, $4 \times 10^2$ cells/100 mL in the effluent of HAp, and $1 \times 10^5$ cells/100 mL in the effluent of H50p (Fig. 5). The abundances were of the same order of magnitude as those found previously for the same systems (Headley et al., 2013). Along the flow path of H50p, there was a steady decline in the number of culturable *E. coli*. In the first portion of HAp, the counts of viable *E. coli* decreased similarly to H50p. Then, at the middle of the wetland where the water chemistry changed substantially, there was a reduction by about 2 log units followed

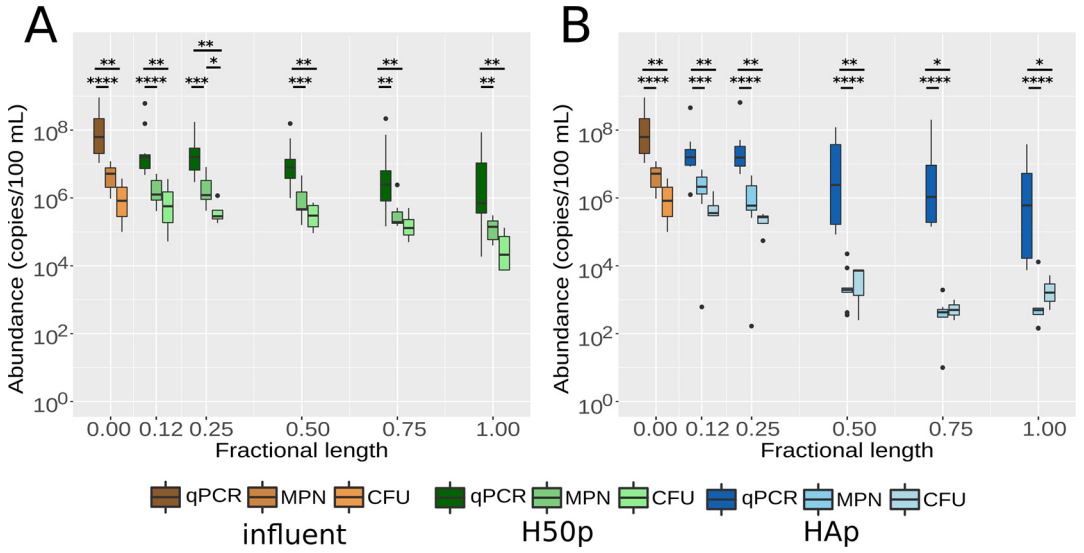


**Fig. 5.** Abundance of *Escherichia coli* along the flow path of the treatment wetlands. A) Influent (brown) and H50p (green). B) Influent (brown) and HAp (blue). Abundance per 100 mL water sample was measured by qPCR (dark colour tones, $n_{qPCR} = 12$ sampling days), most probable number (MPN, intermediate colour tones, $n_{MPN} = 9$ sampling say) and colony forming units (CFU, light colour tones, $n_{CFU} = 4$ sampling days). Statistical significance was calculated with Wilcoxon test (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

by a further slight decrease in the remainder of the wetland. A different picture emerged from measurements of *uidA* copy numbers. In both systems, there was an essentially steady decline in *uidA* numbers, starting with on average $7 \times 10^7$ copies/100 mL. Thus, in H50p the percentage of total *E. coli* that could be cultured was constant along the flow path at approximately 10 %. In contrast, in HAp the proportion of viable *E. coli* decreased from about 10 % to 0.1 % at the middle of the system. We think the best explanation for the different patterns in HAp and H50p is a substantial loss of *E. coli* viability at about the middle of HAp, likely due to oxidative stress and/or starvation.

## 4. Conclusions and outlook

The results of this study argue against a substantial effect of sulfonamides at the in situ concentrations (<100 ng/L) in the investigated treatment wetlands on the prevalence of *sul* genes. Instead, we provide evidence that the level of integrase expression and activity of class 1 integrons is elevated by cellular stress, in particular in the investigated aerated wetland. Higher integrase activity affects the arrangement of AR gene cassettes in class 1 integrons, resulting in a greater integron diversity in microbial isolates from the first half of HAp. The results exemplify that the distribution potential of an AR gene when linked to a mobile genetic element is inherently affected by that element. Thus, a change in ambient conditions unrelated to antimicrobials can be a driver for AR gene mobility if the carrying mobile genetic element responds to that change.

It is important to note that the diversity of class 1 integrons with multiple AR gene cassettes among the isolates and the prevalence of *intI1* and *sul* genes increased only transiently in the aerated wetland. The integron diversity and abundance of all AMR indicators relative to 16S rRNA gene copy numbers did not change when comparing influent versus effluent. The effluent of the aerated treatment wetland, HAp, had about equal of even lower abundance of the investigated AMR indicators as that of activated sludge treatment systems. Apparently, the numerical reduction of members from the influent microbial community in the treatment wetland contributed strongly to the prevalence attenuation of the indicators. The performance of HAp is remarkable because it is achieved at substantially lower capital and operational costs than that of activated sludge treatment systems.

Further insights into the effect of aeration during wastewater treatment on the fate of class 1 integrons and associated AR genes could be obtained in the future by measuring stress level of individual cells, e.g. via recording indicators for starvation and oxidative DNA damage. We envision that an analsis at single-cell resolution would be highly advantageous, as a heterogeneous decrease in viability at the population level may confound the results. Single-cell analysis could also allow for assessing how much of the observed transient increase of *intI1* and *sul* in the aerated wetland was due to gene multiplication within individual cells, or to horizontal transfer to new bacterial hosts via mobile elements such as conjugative plasmids. The latter possibility is more critical when considering the One Health aspect of AMR.

## CRediT authorship contribution statement

**Camila A. Knecht:** Conceptualization, Formal analysis, Methodology, Investigation, Writing – original draft. **Markus Krüger:** Methodology, Investigation. **Simon Kellmann:** Methodology, Investigation. **Ines Mäusezahl:** Methodology, Investigation. **Monika Möder:** Methodology, Investigation. **Olawale O. Adelowo:** Writing – review & editing, Funding acquisition. **John Vollmers:** Formal analysis, Methodology, Investigation. **Anne-Kristin Kaster:** Formal analysis, Supervision, Funding acquisition. **Jaime Nivala:** Writing – review & editing, Supervision. **Jochen A. Müller:** Writing – review & editing, Conceptualization, Supervision, Funding acquisition, Project administration.

## 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.2022.157318.

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