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Identification of Antibiotic Resistance Gene Hosts in Treatment Wetlands Using a Single-Cell Based High-Throughput Approach

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Abstract: Determining the prevalence of antimicrobial resistance (AMR) in non-clinical settings is vital for better management of the global AMR crisis. Untreated and even treated wastewaters are important sources that release AMR into the environment. Methodologically, it is difficult to generate a comprehensive in situ profile of antibiotic resistance gene hosts. Here, we used epicPCR (emulsion, paired isolation, and concatenation PCR) as a cultivation-independent method to reveal the host profiles of the AMR indicator genes *intI1*, *sul1*, *sul2*, and *dfrA1* in two constructed wetlands treating municipal wastewater. Overall, the epicPCR analysis revealed a profile of AMR indicator gene hosts that is consistent with literature data from cultivation-based approaches. Most carriers of antibiotic resistance (AR) genes and likely of class 1 integrons belonged to the *Gammaproteobateria*, particularly the *Burkholderiaceae* and *Rhodocyclaceae* families, followed by members of the *Campylobacterota*, *Desulfobacterota*, and *Firmicutes*. The analysis also identified several novel hosts for the indicator genes widely distributed in the wetlands, including the genera *Legionella* and *Ralstonia*. Therefore, the application of epicPCR has produced an expanded insight into the in situ indicator gene host profile, while highlighting the role of the environment as a reservoir for AMR.

Keywords: antimicrobial resistance; wastewater; constructed wetland; single-cell analysis; epicPCR

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1. Introduction

Infectious diseases caused by antibiotic-resistant bacteria are one of the major health burdens of our time. To contain the spread of these bacteria and the antibiotic resistance (AR) genes they carry (collectively, antimicrobial resistance or AMR), the One Health approach advocates for the inclusion of environmental compartments in surveillance and prevention efforts [1,2]. The release of treated and untreated wastewater into surface waters is a substantial pathway for the distribution of AMR via the environment [3,4]. Monitoring the prevalence of AMR in wastewater is therefore an important part of evaluating the risk to human and animal health posed by the spread of AMR in the environment [5].

The antibiotic resistance genes that are commonly detected in waste and surface waters include *sul1*, *sul2*, and *dfrA1*, all of which confer resistance against inhibitors of folate biosynthesis [3,6]. The *sul1* gene is part of the classical class 1 integron, an important carrier in AR gene dispersal [7]. Another defining feature of that integron type is the *intI1* gene, which codes for an integrase that can recombine the AR gene cassette content of the integron. The association of *intI1* with AMR has led to its use as an indicator of anthropogenic pollution in water bodies [8,9]. Class 1 integrons have predominantly been found in *Gammaproteobacteria* [10]. A recent publication listed 104 bacterial species from 44 genera as carriers of that integron [11]. Two thirds of the genera belong to the *Pseudomonadota* (almost always *Gammaproteobacteria*), with the remainder being members of the *Actinobacteriota*, *Bacteroidota*, *Campylobacterota*, and *Firmicutes*. *Gammaproteobacteria* also account for by far the largest proportion of integrons detected in sequence databases. Approximately one fifth of all sequenced genomes of this class contain at least one integron of the class 1 or a similar type [12]. Yet, the information on prevalence in sequenced genomes does not necessarily translate 1:1 to a specific environmental site. Generating a comprehensive in situ host profile of class 1 integrons and AR genes, including *sul1*&*2* and *dfrA1*, is not a trivial task. The classical cultivation-dependent approach is subject to bias, as many bacteria do not grow readily in the laboratory. Shotgun metagenomics, as a cultivation-independent approach, can detect AR genes in a microbial community, but has limitations in assigning respective hosts due to the frequent association of these genes with mobile genetic elements [13–15]. Host profiling can be achieved using recently developed molecular biology methods. Proximity DNA ligation methods such as Hi-C involve the metagenomic sequencing of DNA that has been cross-linked prior to extraction from its host [15]. However, the generation of one metagenome per sample renders the approach expensive if the research aim is to capture temporal and spatial variations in host profiles. An alternative method is epicPCR (emulsion, paired isolation, and concatenation polymerase chain reaction), which generates fusion PCR amplicons of parts of the 16S rRNA gene and another target gene of single cells imbedded in polyacrylamide beads [16]. The fusion products are then isolated and sequenced in parallel. So far, the method has been applied to reveal bacterial hosts of various genes associated with AMR, including *intI1* and *sul1*, in environmental samples [17–21]. A variant of epicPCR was used to identify bacterial hosts of beta-lactamases in human gut microbiomes [22].

Here, we used epicPCR to identify hosts of *intI1*, *sul1*&*2*, and *dfrA1* in two treatment wetlands. Such wetlands are nature-based solutions that are increasingly applied as a cost-effective option to treat domestic and industrial wastewater [23,24]. Removal of bacteria and chemical pollutants introduced with the influent, including antibiotics, can be achieved with similar or even better efficiency than activated sludge-based treatment systems, although with longer hydraulic residence times [25–27]. In a previous study on the fate of AMR in the same treatment wetlands investigated herein, we observed increases in integron integrase activity and *intI1* and *sul* abundance, which were likely caused by cellular stress [28]. There was no evidence of an influence of folate biosynthesis inhibitors on the prevalence of AMR. Increases in *intI1* and *sul1* abundance, apparently independent of such inhibitors, have also been detected in rivers downstream of wastewater treatment plants [29–31]. It is uncertain which bacteria are involved in such increases. In the wetlands, we found mostly *Aeromonas* spp., *Escherichia coli*, and *Pseudomonas* spp., as hosts of the indicator genes, using cultivation-dependent profiling. The present application of epicPCR aimed to further explore the host profiles.

2. Materials and Methods

2.1. Study Site and Sampling Regime

The pilot-scale horizontal subsurface flow treatment wetlands investigated in this study are located at the Langenreichenbach Ecotechnology Research Facility, 42 km north-

east of Leipzig, Germany (51.50613 N, 12.89758 E). The design and operation of the wetlands have previously been described in detail [32]. In brief, the wetlands have a surface area of 5.6 m^2 and are named H50p for Horizontal, 50 cm depth, and planted, and HAp for Horizontal, Aerated, and planted. Their main vegetation is the common reed, *Phragmites australis*. Wastewater from the surrounding rural residential villages is pretreated in a septic tank and pumped from there to H50p and HAp, as the sole source of water other than precipitation.

Samples were taken monthly from May through July 2017 for method development, and in August 2017 for data generation. The conventional water characteristics (temperature, pH, dissolved oxygen, and inorganic nitrogen species) and relevant microbiological data (AR gene abundances and 16S rRNA profiles) of the sampling period have been previously reported [28,33]. There was only small variation in the conventional and microbiological water characteristics during the sampling period. One-liter samples were taken from the septic tank, the effluent, 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 samples were chilled and transported within 1–2 h to the laboratory for further processing.

2.2. epicPCR

The following protocol is based on previous studies with some modifications [16,18]. Table S1 shows all of the primers used for fusion PCR, (semi)-nestedPCR, and the introduction of adapter sequences for Illumina sequencing [34–37]. Almost all primers were previously used either in epicPCR or in quantitative PCR. The exception was the forward primer for the amplification of *dfrA1*, which was newly designed using NCBI's online tool Primer-BLAST, with the reference sequence downloaded from GenBank (accession number 3244905). The establishment of the overall method, including optimized PCR conditions, was performed using a *Citrobacter* sp. strain S22 isolated from the treatment systems for *sul1*, *sul2* and *dfrA1* [28], and *Pseudomonas plecoglossicida* M135 for *intI1* [38]. Cell numbers in the assays were determined using a Coulter counter (Beckman Coulter, Indianapolis, IN, USA). Correct amplification of the intended epicPCR fusion product was ensured via agarose gel electrophoresis and Sanger sequencing (Macrogen, Amsterdam, The Netherlands).

Samples were first inspected via microscopy. When cell aggregates were present, samples were passed through a cell strainer (40 µm pore size; Falcon, NY, USA). Cells in all samples were counted via DAPI (4′,6-diamidin-2-phenylindol) staining using a fluorescence microscope (Axioscope, Zeiss, Oberkochen, Germany), and then pelleted in a benchtop centrifuge (Eppendorf, Hamburg, Germany) at 8000 rpm for 15 min at room temperature. The volume of the respective sample depended on the sampling site, and ranged from 2 to 180 mL to obtain approximately 1–2 × 10^8 cells per 100 µL epicPCR assay. Cell numbers were determined with a Coulter counter. After centrifugation, the pellets containing the cells were carefully resuspended using vortexing and pipetting in 100 µL of nuclease free water. The absence of cell aggregates in the suspension was checked for using microscopy. Then, the cell suspension was amended with 100 µL of 30% acrylamide/bis-acrylamide and 25 µL of ammonium persulfate (APS), and gently vortexed. After addition of 600 µL mineral oil, the mixtures were vortexed for 30 s at 3000 rpm. Then, 25 µL of tetramethylethylenediamine (TEMED) was added, followed by vortexing for 45–60 s at 3000 rpm. The mixture was kept at room temperature for 90 min to allow polymerization. To collect the polyacrylamide beads, 800 µL of diethyl ether was added, whereupon a precipitate was formed. After removing the diethyl ether and adding 1 mL of nuclease-free water, mixing, and centrifuging for 30 s at 12,000× *g*, three layers formed: a bottom layer containing the beads, a middle layer containing a mixture of oil and water, and an upper layer of oil. The oil layer was discarded. This step of mixing water into the bead suspension was repeated until no more three-layer-formation was visible. The water layer was discarded and 1 mL of TK buffer (20 mM Tris-HCl, pH 7.5,

60 mM KCl) was added to the beads. The bead suspension was passed through a cell strainer (40 μm pore size) with the flow-through containing the beads. The absence of cell aggregates inside the beads was checked for using microscopy.

For cell lysis, the beads were distributed into 250 μL aliquots; 2 μL of Ready-Lyse™ Lysozyme Solution (4 × 10^6 U, Cambio, Cambridge, UK) was added to each aliquot and incubated overnight at 37 °C. Aliquots were pooled and centrifuged at 12,000× *g* for 30 s. The supernatant was discarded. Then, the TK buffer was used to obtain a total volume of 400 μL in which the beads were resuspended. Next, 100 μL of proteinase K (>600 U/mL, Thermo Fisher Scientific, Waltham, MA, USA) and 4 μL of Triton X-100 were added to the beads and incubated at 37 °C for 30 min and 95 °C for 10 min. Samples were aliquoted into 1 mL and centrifuged at 12,000× *g* for 30 s. The beads were resuspended with TK buffer to obtain a total volume of 500 μL.

For fusion PCR, 90 μL of the polyacrylamide beads, four 2 mm glass beads, 900 μL ABIL emulsion oil (Evonik, Essen, Germany), and 110 μL of the following mix was added per microcentrifuge tube: 40 μL of HF buffer for S7 Fusion High-Fidelity DNA Polymerase (Mobidiag), 5 μL of 10 mM dNTPs, 20 μL of primer mixture (forward and reverse primers at 10 μM, fusion primer at 0.1 μM), and 16 μL of S7 Fusion High-Fidelity DNA Polymerase (Mobidiag, Espoo, Finland). As in Hultman et al. [18], blocking primers [16] were not used, because unwanted chimeras could not be detected via gel electrophoresis and Sanger sequencing using our final fusion PCR conditions for the four AMR indicator genes. The fusion PCR mixture was emulsified by vortexing at 3000 rpm for 1 min and aliquoted into 60 μL. The PCR program run was as follows: 98 °C for 30 s, 25 cycle of [98 °C for 5 s, 55 °C for 30 s, 72 °C for 30 s], 72 °C for 5 min. After PCR, 2 μL of 50 mM of ethylenediaminetetraacetic (EDTA) was added.

To break up the beads and purify the PCR fusion products, the bead suspensions were centrifuged at 13,000× *g* and 22 °C for 5 min, and the upper phase was discarded. To each bead sample, 1 mL of diethyl ether was added and vortexed gently. The suspensions were centrifuged for 1 min at 13,000× *g* and the upper phase was discarded. This step was repeated. This was followed by extraction with ethyl acetate and two more extractions with diethyl ether. Bead extractions were left open in a laminar flow hood for 10 min. Then, 100–150 μL of the lower phase was taken to purify the fusion PCR products using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Quality and quantity controls were carried out through agarose gel electrophoresis, spectrophotometry (NanoDrop 1000, NanoDrop Technologies, Wilmington, DE, USA), and Sanger sequencing of selected products.

Then, the fusion products were subjected to (semi)-nested PCR. For the *sul* genes, a semi-nested approach was used, while a nested approach was applied for *dfrA1* and *intI1*. Different volumes (4–6 μL) of the fusion products were added to the following mix: 5 μL of 5X HF buffer, 0.5 μL of 10 mM dNTP, 1.5 μL of 10 μM of the primers, 0.25 μL of 2 U/μL of S7 Fusion Polymerase, 15.5 μL ddH_2O. PCR was performed following a cycling program: 98 °C for 30 s, depending on the target of 30–40 cycles of [98 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s], and a final elongation step of 5 min at 72 °C. The PCR products were cleaned-up using ethanol precipitation and dissolved in 13 μL of EB buffer. The quality and quantity of the products were again checked via agarose gel electrophoresis, spectrophotometry, and Sanger sequencing of selected products.

2.3. Illumina Sequencing and Data Analysis

Illumina sequencing adapters were added in seven PCR cycles with NEBNext Multiplex Oligos, using a NEBNext Ultra II FS DNA Library Prep kit. PCR products were purified with SPRI beads (Beckman Coulter). Quantity controls were carried out using the Quan-iT PicoGreen dsDNA Assay Kit (Roche Diagnostics, Meylan, France) and a Quanti-Fluor-ST Fluorometer (Promega, Madison, WI, USA), while purity was assessed with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Paired-end sequencing (2 × 300 bp) was carried out using an Illumina MiSeq-System (Illumina, San

Diego, CA, USA). Illumina reads were analysed with fastQC [39] to check their quality. Forward and reverse reads were analysed individually. Forward reads were used to confirm the AMR part of the fusion product, and reverse reads were used for phylogenetic analysis. Sequencing data were analysed in R using the packages DADA2 [40], phyloseq [41], Biostrings [42] and ggplot2 [43]. Reverse raw reads containing 16S rRNA gene sequences were 300 nt long on average. Their quality was visualized with the function plotQualityProfile from the DADA2 package. All reverse reads were then trimmed to remove the primer and adapter sequence (24 nt) and truncated to 150 nt, because in some samples, the quality substantially decreased from that position onwards. Phylogenetic assignments are based on the Genome Taxonomy Database (GTDB) [44], with two exceptions, in which older synonyms and classifications are used for familiarity (*Firmicutes* as the phylum name and *Comamonadaceae* as family name).

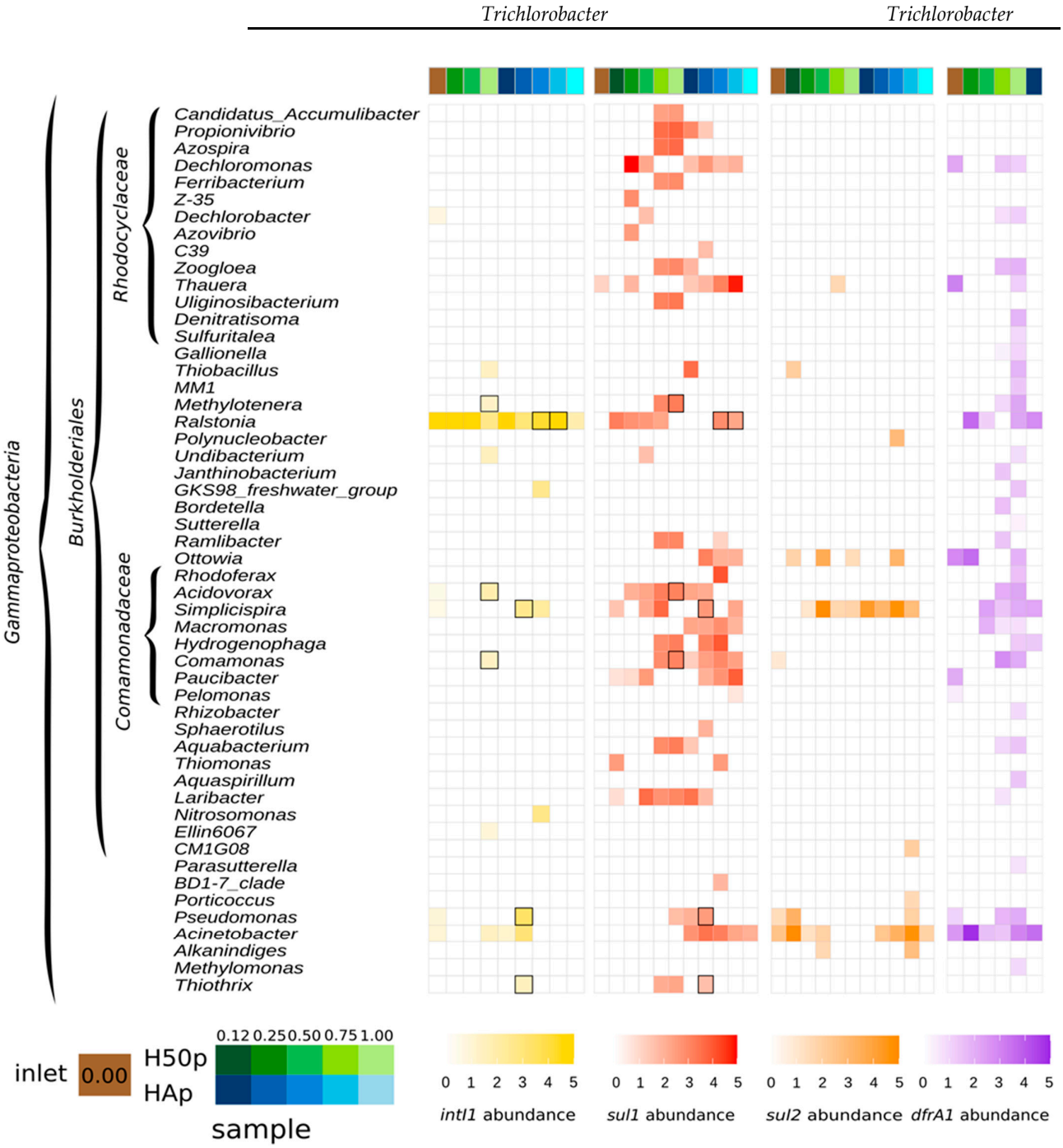
3. Results and Discussion

3.1. AMR Gene Hosts along the Watercourse of the Treatment Wetlands

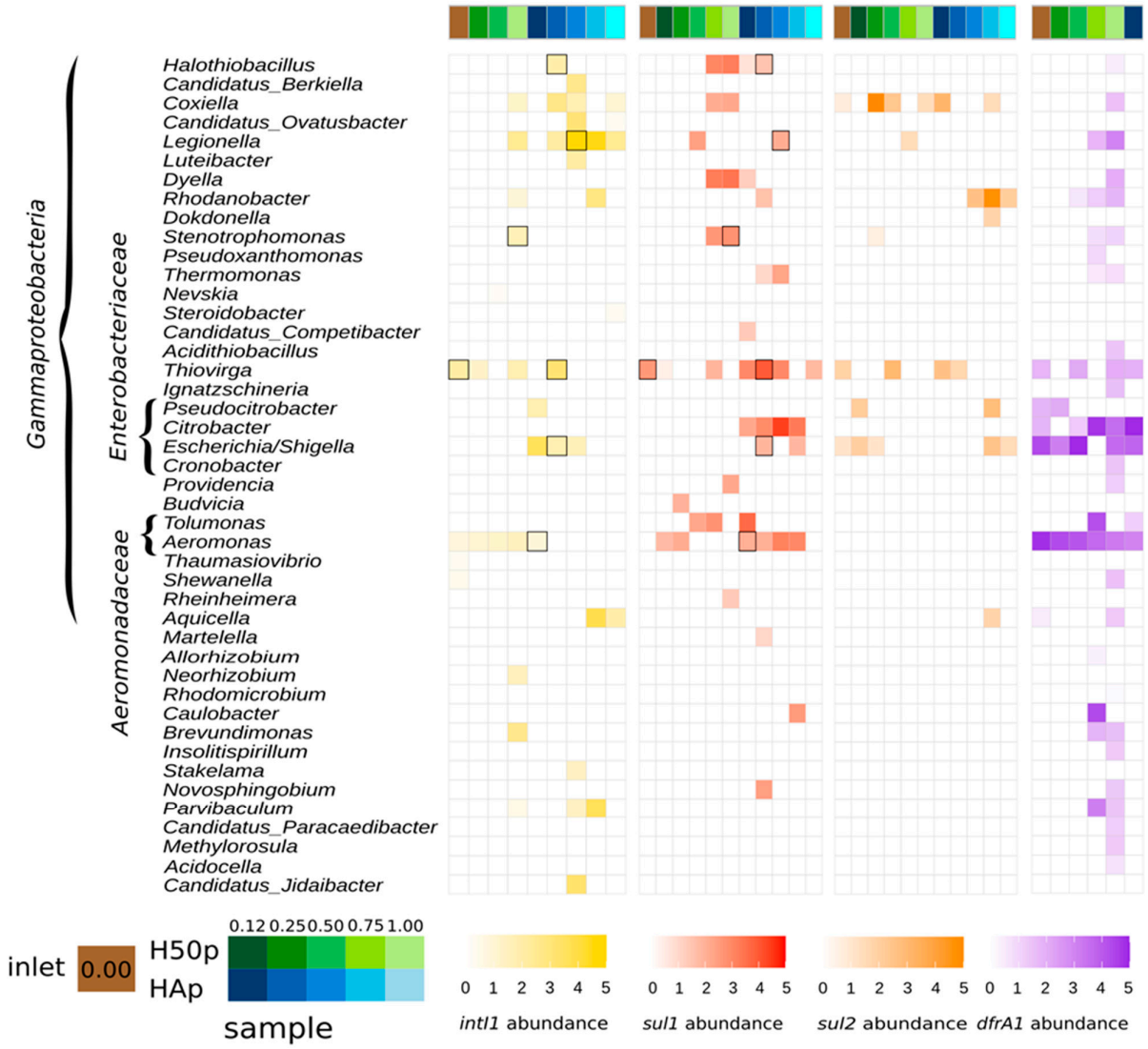
EpicPCR is semi-quantitative because it has the same biases as standard PCR, such as a different strength of primer binding to the target sequence, and there are taxon-specific differences in cell lysis efficiency. Therefore, in the following, we focus on the qualitative aspects of the results, i.e., the detection of specific AMR gene host rather than their presumptive abundance in the treatment wetlands. Relative abundances are nevertheless indicated in Figure 1A–D. In epicPCR analysis, false positive results can occur when cells with and without the target gene are present in the same polyacrylamide bead [16,45]. Since millions of beads are generated per epicPCR assay, such instances may be overlooked during microscopic inspection, as carried out here. In their study on improvements in epicPCR, Roman et al. [45] pointed out that it is currently impossible to differentiate between false positive artifacts and rare taxa found only in few samples. They assigned confidence levels according to the taxa detection frequency in biological replicates. We adapted their approach by showing all detected genera in Figures 1A–D, and listing genera found at more than 50% of the sampling sites in Table 1; this prevalence we consider a high-confidence detection. Only genera detected at two or more sampling sites are specifically discussed in the following (except for members of the *Archaea*, see Section 3.3).

Table 1. Genera found at >50% of the sampling sites as hosts of AMR indicator genes.

intI1	***sul1***	***sul2***	***dfrA1***
	Acidovorax		
	Acinetobacter	*Acinetobacter*	*Acinetobacter*
Aeromonas	*Aeromonas*		*Aeromonas*
Bdellovibrio			
			Citrobacter
	Commamonas		
	Coxiella	*Coxiella*	
	Dechloromonas		
			Escherichia/Shigella
	Laribacter		
Legionella			
	Paucibacter		
	Protocatella		
Ralstonia	*Ralstonia*		*Ralstonia*
	Simplicispira	*Simplicispira*	*Simplicispira*
	Subdoligranulum	*Subdoligranulum*	
	Thauera		
Thiovirga	*Thiovirga*		*Thiovirga*


Trichlorobacter
Trichlorobacter
Gammaproteobacteria
Burkholderiales
Rhodocyclaceae
Comamonadaceae
Candidatus_Accumulibacter
Propionivibrio
Azospira
Dechloromonas
Ferribacterium
Z-35
Dechlorobacter
Azovibrio
C39
Zoogloea
Thauera
Uliginosibacterium
Denitratisoma
Sulfuritalea
Gallionella
Thiobacillus
MM1
Methylotenera
Ralstonia
Polynucleobacter
Undibacterium
Janthinobacterium
GKS98_freshwater_group
Bordetella
Sutterella
Ramlibacter
Ottowia
Rhodoferax
Acidovorax
Simplicispira
Macromonas
Hydrogenophaga
Comamonas
Paucibacter
Pelomonas
Rhizobacter
Sphaerotilus
Aquabacterium
Thiomonas
Aquaspirillum
Laribacter
Nitrosomonas
Ellin6067
CM1G08
Parasutterella
BD1-7_clade
Porticoccus
Pseudomonas
Acinetobacter
Alkanindiges
Methylomonas
Thiothrix
inlet
0.00
H50p
HAp
0.12 0.25 0.50 0.75 1.00
sample
0 1 2 3 4 5
intI1 abundance
0 1 2 3 4 5
sul1 abundance
0 1 2 3 4 5
sul2 abundance
0 1 2 3 4 5
dfrA1 abundance

(**A**)


Gammaproteobacteria
Enterobacteriaceae
Aeromonadaceae
Halothiobacillus
Candidatus_Berkiella
Coxiella
Candidatus_Ovatusbacter
Legionella
Luteibacter
Dyella
Rhodanobacter
Dokdonella
Stenotrophomonas
Pseudoxanthomonas
Thermomonas
Nevskia
Steroidobacter
Candidatus_Competibacter
Acidithiobacillus
Thiovirga
Ignatzschineria
Pseudocitrobacter
Citrobacter
Escherichia/Shigella
Cronobacter
Providencia
Budvicia
Tolumonas
Aeromonas
Thaumasiovibrio
Shewanella
Rheinheimera
Aquicella
Martelella
Allorhizobium
Neorhizobium
Rhodomicrobium
Caulobacter
Brevundimonas
Insolitispirillum
Stakelama
Novosphingobium
Parvibaculum
Candidatus_Paracaedibacter
Methylorosula
Acidocella
Candidatus_Jidaibacter
inlet
0.00
H50p
HAp
0.12 0.25 0.50 0.75 1.00
sample
0 1 2 3 4 5
intI1 abundance
sul1 abundance
sul2 abundance
dfrA1 abundance

(**B**)


Desulfobacterota
Firmicutes
Syntrophus
Smithella
Pseudopelobacter
Trichlorobacter
Desulforhopalus
Desulfoprunum
Desulfobulbus
Desulfonatronobacter
Desulfococcus
Desulforegula
Desulfobacter
Desulforhabdus
Desulfomonile
Desulfomicrobium
Desulfovibrio
Peredibacter
Bdellovibrio
Silvanigrella
Amnipila
Anaerovorax
Family_XIII_AD3011_group
Mogibacterium
Acidaminobacter
Fusibacter
Romboutsia
Peptostreptococcus
Proteocatella
Acetoanaerobium
XBB1006
Lachnoclostridium
Blautia
Anaerostipes
Lachnospira
Clostridium_sensu_stricto_8
Proteiniclasticum
Clostridium_sensu_stricto_14
Clostridium_sensu_stricto_1
NK4A214_group
Intestinimonas
Saccharofermentans
Anaerofilum
Subdoligranulum
Ruminococcus
Christensenellaceae_R-7_group
Lactobacillus
Trichococcus
Enterococcus
Streptococcus
Vulcanibacillus
Nosocomiicoccus
Erysipelotrichaceae_UCG-003
Pelosinus
Anaerosinus
Succinispira
Phascolarctobacterium
TC1
Dehalobacterium
inlet
0.00
H50p
HAp
0.12 0.25 0.50 0.75 1.00
sample
0 1 2 3 4 5
intI1 abundance
0 1 2 3 4 5
sul1 abundance
0 1 2 3 4 5
sul2 abundance
0 1 2 3 4
dfrA1 abundanc

(**C**)

(**D**)

Figure 1. Genera detected as hosts of *intI1* (yellow squares), *sul1* (red), *sul2* (orange), and *dfrA1* (purple) in samples from the common inlet (brown) and the two treatment wetlands H50p (green) and HAp (blue). Relative abundances are Log10 normalized (Roman et al., 2021), indicated per color intensity. (**A**) Hosts belonging to the *Gammaproteobacteria*, including members of the families *Rhodocylaceae* and *Comamonadaceae*. (**B**) Further hosts belonging to the *Gammaproteobacteria* and other *Proteobacteria*. (**C**) Hosts belonging to the *Desulfobacterota*, *Bdellovibrionota*, and *Firmicutes*. (**D**) Hosts belonging to the *Campylobacterota*, *Bacteroidota*, and *Archaea*.

After quality filtering, we obtained 850,050 to 1382,015 clean reads for the four genes. Targeting *dfrA1* in HAp yielded high-quality results only for the first sampling site, which does not match the previous detection of the gene along the entire flow path of the wetland [28]. Establishing epicPCR for *dfrA1* was more difficult in our laboratory than for the other indicator genes, and included the application of a new forward fusion primer. Further optimization work for *dfrA1* is apparently needed in the future. In total, 209 genera were identified as carrying at least one of the AMR indicator genes in at least one sample (Figure 1A–D). Previously, ten genera harboring the studied genes were isolated from both treatment wetlands and the common inflow: *Acinetobacter*, *Aeromonas*, *Arcobacter*, *Citrobacter*, *Escherichia*, *Flavobacterium*, *Pseudomonas*, *Providencia*, *Shewanella*, and *Stenotrophomonas* [28]. All of these were also found in this study to be carriers of at least one of the target genes (with the qualifier that the Illumina reads did not allow for differentiation between *Escherichia* and *Shigella*). About half of all the detected genera (96/209) belonged to the *Pseudomonadota* (Figure 1A,B), especially to the gammaproteobacterial order *Burkholderiales*, which is consistent with reports on the taxonomic distribution of these indicator genes [10–12]. Among the *Burkholderiales*, there were 14 members of the *Rhodocyclaceae* that carried *sul1*, with some also harboring *dfrA1* (Figure 1A). Members of this family are frequently found in WWTP systems [46]. Recently, a conjugative IncP-1 type plasmid with a class 1 integron was discovered in the genome of the *Rhodocyclaceae* genus *Thauera* [47]. This genus was detected as carrier of *sul1* at 6 out of 11 of our sampling sites, supporting the hypothesis that *Rhodocyclaceae* may constitute a substantial reservoir of antibiotic resistance genes in WWTP systems [47]. Furthermore, 21 genera of the *Burkholderiaceae*, particularly close relatives of *Comamonas* (i.e., members of the family *Comamonadaceae,* according to the SILVA taxonomy), were found to carry *sul1* and *dfrA1,* and to a lesser extent, *intI1* and *sul2* (Figure 1A). These families are known to harbor the AMR indicator genes [48]. *Burkholderiaceae* were also among the most common hosts of *sul1* in a recent epicPCR study investigating WWTP [20]. Interestingly, the genus *Ralstonia*, a member of the *Burkholderiaceae*, was among the most prevalent hosts of *int1*, *sul1*, and *dfrA1* in both treatment wetlands (Figure 1A and Table 1). To our knowledge, these genes have previously been found only in a few isolates and genomes of *Ralstonia* spp. [29,49]. The present finding is significant because several *Ralstonia* spp. are opportunistic pathogens that can be prevalent in drinking water sources [50,51]. Moreover, the folate biosynthesis inhibitors sulfamethoxazole and trimethoprim are among the best treatment options for *Ralstonia* spp. infections [50]. The last member of the *Burkholderiales* that we detected and would like to highlight is *Laribacter* (family *Aquaspirillaceae*), which was a carrier of *sul1* in both HAp and H50p. Class 1 integrons and *sul2* have been found in sequenced genomes of *Laribacter hongkongensis* [52], a potential cause of infectious diarrhea [53].

In addition, we found seven taxa of the gammaproteobacterial order *Pseudomonadales*, including *Pseudomonas*, carrying the AMR indicator genes in the wetlands (Figure 1A). Numerous *Pseudomonas* spp. strains carrying class 1 integrons have been described in the literature, and respective isolates were obtained from HAp and H50p throughout the flow path [28]. All four genes were also found in association with *Acinetobacter* (family *Moraxellaceae*). *Acinetobacter* spp. are ubiquitous in environmental habitats as well as in animals and humans, and can cause various diseases [54]. Etiological species include *A. baumannii*, a member of the ESKAPE group, which are six virulent and antibiotic-resistant bacterial pathogens that cause critical nosocomial infections [55]. There are numerous sequenced *Acinetobacter* spp. isolates with *dfrA1* and *sul* resistances genes and class 1 integrons [56–59]. Our findings are in line with the notion that the environment is a substantial reservoir of antibiotic-resistant *Acinetobacter* spp. [54].

Furthermore, we discovered four taxa that have been described as symbionts of amoebae, namely *Coxiella*, Candidatus Berkiella, Candidatus Ovatusbacter, and *Legionella* (Figure 1B), the latter being the etiological agent of legionellosis. These taxa are not known to be typical carriers of the AMR indicator genes. In GenBank, we found only a partial *sul2* in a sequenced *L. pneumophila strain from* hospital water associated with the 2016

outbreak of legionellosis in Flint, MI, USA [60]. Although folate biosynthesis inhibitors are currently not the drugs of choice for the treatment of legionellosis, the potential presence of class 1 integrons in the genus (see also Section 3.2) is of concern.

Members of the *Enterobacteriaceae* are well-known carriers of the four AMR indicator genes. Here, we found *Escherichia*/*Shigella* to be associated with each of the four genes, and other members of the *Enterobacteriaceae* with some of these genes (Figure 1B). Previously, each AMR indicator gene was detected in isolates of these taxa from both treatment wetlands [28]. While the epicPCR results match the literature data on host distribution of the AMR genes, they also demonstrate the detection limitation of the method. For example, the *Citrobacter* sp. strain used to develop the method for *sul1*, *sul2* and *dfrA1* was isolated from the wetlands, but epicPCR provided evidence only for *Citrobacter* spp. with *sul1* and *dfrA1,* but not with *sul2,* in the treatment systems. Since certain hosts were not always detected despite being present, we did not attempt to estimate the horizontal gene transfer (HGT) of the AMR indicators, as has been published elsewhere [20]. Reliable evaluation of HGT would have required the analysis of more replicates, which was restricted by the labor required and the high costs of Phusion DNA polymerase.

Furthermore, epicPCR revealed the genus *Aeromonas* (family *Aeromonadaceae*) to be carriers of *intI1* and *sul1* at half of the sampling sites, and of *dfrA1* at all sites (Figure 1B). No fusion product with *sul2* was detected at any of the sites, although the gene was previously detected in nine distinct *Aeromonas* spp. isolates recovered from both wetlands [28]. Members of the genus *Aeromonas* are ubiquitous in freshwater habitats and can be also found in the clinical environment, and have been identified as carriers of the four AMR indicator genes [61,62]. Moreover, *Aeromonadaceae* were inferred in a Hi-C study to be a particularly prevalent reservoir of AR genes in a wastewater treatment plant [15].

Other carriers of the four genes in the wetlands belonged to the *Desulfobacterota*, including *Trichlorobacter* (also classified as *Geobacter*) and various sulfate-reducing bacteria, *Bdellovibrionota* (*Bdellovibrio* as carrier of *intI1*), and 39 genera of *Firmicutes* (Figure 1C). *Firmicutes* are known to harbor class 1 integrons [11]. Here, the detection of multiple *Firmicutes* genera is noteworthy, as these are Gram-positive bacteria for which the lysis of encapsulated single cells is often difficult. Incubation of polyacrylamide beads with Ready-Lyse lysozyme may have improved cell lysis [22]. Furthermore, members of the *Campylobacterota* were particularly prevalent, represented by the family *Arcobacteraceae* (*Arcobacter*, *Poseidonibacter*, *Pseudoarcobacter*) and the genera *Sulfuricurvum*, *Sulfurimonas*, *Sulfurospirillum*, and *Sulfurovum* (Figure 1D). The latter are sulfur-oxidizing bacteria that can occur in high absolute and relative abundance in the rhizosphere of wetland plants [63]. To our knowledge, they have not been previously identified as carriers of antibiotic resistance genes and class 1 integrons. In other isolated members of the *Campylobacterota* such as *Campylobacter* spp., class 1 integrons with various AR gene cassettes are commonly detected [64]. *Campylobacteraceae* including *Arcobacter* were also previously detected by epicPCR as hosts of *intI1* and *qacEΔ1* in the inflow of two urban WWTPs [18].

3.2. Potential Class 1 Integron Carriers in the Treatment Wetlands

Next, we searched for identical 16S rRNA fragments among the sequenced *intI1* and *sul1* fusion products from a given sample. While this approach does not provide conclusive evidence that a respective taxon at the sampling site contains at least one cell harboring a classical class 1 integron, it is nevertheless a good indication thereof. In total, we found identical 16S rRNA fragments belonging to 22 genera, indicated by black boxes in Figure 1A–D. The taxonomic pattern at the phylum level was quite similar to that described in the literature [11]. Thirteen (59%) of the genera belonged to the *Gammaproteobacteria*, seven genera to the *Campylobacterota*, and one each to the *Bacteroidota* and *Desulfobacterota*. Six of the *Gammaproteobacteria* genera have been frequently reported to be carriers of class 1 integrons, including *Aeromonas*, *Escherichia*/*Shigella*, and *Pseudomonas*, representatives of which had previously been isolated from the treatment wetlands [28]. The seven gammaproteobacterial genera that are not or only rarely described in the literature

as carriers of class 1 integrons include *Ralstonia* and *Legionella*, which are among the taxa identified with high confidence (Table 1) and are briefly discussed in Section 3.1. Furthermore, *Paludibacter*, a member of the *Bacteroidota* discovered here, was recently suggested to be a strong candidate host of *intI1* in a lake–river–lake system in Northern Italy [65].

3.3. Evidence for AMR Genes in Methanogenic Archaea

There were also epicPCR fusion products of *sul1* and *dfrA1* with 16S rRNA fragments of methanogenic *Archaea* in three samples (H50p at fractional length 0.12 and 1.00, and HAp at fractional length 0.12). Specifically, the *sul1* products were linked with 16S rRNA fragments of *Methanocorpusculum* and *Methanospirillum*, and the *dfrA1* product linked with a 16S rRNA fragment of *Methanobacterium*, all of which are members of the *Methanobacteriota*. Genes coding for tetrahydrofolate-synthesizing enzymes were discovered in some methanogenic *Archaea* [66], but the amino acid sequence identities are only around 30–40% of the products of *sul1* and *dfrA*, and we have not found closer homologs in the sequenced archaeal genomes of GenBank and PATRIC [67]. We note that the epicPCR results need to be corroborated, since there were only three samples were positive for fusion products with archaeal 16S rRNA gene amplicons and only <20 reads each. Nevertheless, our findings are first evidence for inter-domain transfer of *sul1* and *dfrA1* into members of the *Archaea*. Horizontal transference between Archaea and Bacteria has been suggested for multiple other genes [68–70]. Furthermore, integrons, although not of the class 1 type, were recently discovered at a low frequency in metagenome-assembled genomes (MAGs) of *Archaea*, including members of the *Methanobacteriota* [71]. In the same study, it was shown that class 1 integron integrase harbored by an *E. coli* strain could incorporate archaeal gene cassettes into its integron via the archaeal *attC* recombination site. It is reasonable to hypothesize that archaeal integrase can incorporate bacterial gene cassettes, possibly taken up via transformation [72], into archaeal integrons, which could explain the apparent presence of *sul1* and *dfrA1* in the methanogenic *Archaea* observed here. Future epicPCR analysis could employ primers specifically targeting the archaeal 16S rRNA gene.

4. Conclusions

In this study, the epicPCR analysis revealed a profile of AMR indicator gene hosts highly consistent with literature data, including our previous cultivation-dependent investigation of the same treatment wetlands. Most carriers of the genes and apparently also of class 1 integrons belonged to the *Gammaproteobateria*, followed by members of the *Desulfobacterota, Firmicutes* and *Campylobacterota*. The analysis also identified several novel hosts of the indicator genes that were prevalent in the wetlands. Furthermore, the genes were found in genera with members known to cause disease in humans or domesticated animals, but also in genera without such medical or veterinary significance. The presence of the latter group of microorganisms underscores the function of the environment as a reservoir for AMR. Finally, the diversity of gene hosts observed here relates to the question of how best to address the spread of AMR in environmental compartments [3,4]. Limiting emission loads via improved wastewater treatment would be valuable, especially where risks of transmission are currently high [73]. However, we also note that different microbial hosts may have different survival and gene transmission potentials in the environment. This hampers the quantitative assessment of the effectiveness of AMR attenuation through wastewater treatment, as resistance genes may be propagated again, downstream of the treatment system, through vertical or horizontal transmission, if present in a suitable host. We are not the first to advocate this precautionary principle as a guide to better antibiotics stewardship, and hopefully as a pathway to curbing the spread of AMR worldwide [74–76].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15132432/s1, Table S1: Primers used in this study.

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