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Antibiotic Resistance Genes in River Biofilms: A Metagenomic Approach toward the Identification of Sources and Candidate Hosts

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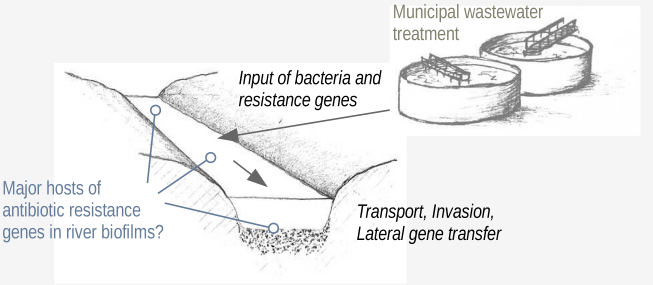
ABSTRACT: Treated wastewater is a major pathway by which antibiotic resistance genes (ARG) enter aquatic ecosystems. However, knowledge gaps remain concerning the dissemination of specific ARG and their association with bacterial hosts. Here, we employed shotgun metagenomics to track ARG and taxonomic markers in river biofilms along a gradient of fecal pollution depicted by crAssphage signatures. We found strong evidence for an impact of wastewater effluents on both community composition and resistomes. In the light of such simultaneity, we employed a model comparison technique to identify ARG−host relationships from nonassembled metagenomic DNA. Hereby, a major cause of spurious associations otherwise encountered in correlation-based ARG−host analyses was suppressed. For several families of ARG, namely those conferring resistance to beta-lactams, particular bacterial orders were identified as candidate hosts. The found associations of *bla*FOX and *cph*A with Aeromonadales or *bla*PER with Chromatiales support the outcome of independent evolutionary analyses and thus confirm the potential of the methodology. For other ARG families including *bla*IMP or *tet*, clusters of bacterial orders were identified which potentially harbor a major proportion of host species. For yet other ARG, like, for example, *ant* or *erm*, no particular host candidates were identifiable, indicating their spread across various taxonomic groups.



KEYWORDS: *antibiotic resistance, bacterial community, biofilm, river, metagenomics*

INTRODUCTION

Antibiotic resistance in pathogenic bacteria poses a global problem of increasing concern.[1] Successful management of the crisis of antibiotic resistance requires a holistic approach that recognizes the particular role of terrestrial and aquatic ecosystems.[2,3] Environmental systems are, on the one hand, an original source of antibiotic resistance genes (ARG).[4,5] On the other hand, they provide reservoirs and pathways for the spread of acquired ARG that emerge and proliferate in conjunction with antibiotic therapies in human healthcare and livestock farming.[6−8]

In particular, the role of treated wastewater emissions on the occurrence of ARG in surface waters was examined in different geographical settings and with a focus on varying resistance determinants.[9−11] Moreover, a number of successful attempts were made to disentangle the impacts of wastewater disposal and other anthropogenic activities on freshwater resistomes.[12−14] The vast majority of those case studies relied on qPCR technology to quantify selected ARG and, possibly, genetic elements involved in their proliferation like, for example, integrons. In recent years, shotgun metagenomics (SMG) is increasingly employed to capture ARG in environmental systems.[15−17] In contrast to alternative methodologies, SMG yields information on the gene pool of entire bacterial communities without prior selection of targets. Thus, SMG potentially detects a broad spectrum of resistance genes, including those not being typically considered in surveillance.[18] Moreover, metagenomic sequencing allows for the simultaneous extraction of information on resistome and community composition from one and the same set of DNA sequences. Finally, relative abundances obtained by SMG are believed to be unbiased since neither cultivation nor PCR are involved which could result in unequal target amplification. At the same time, the lack of amplification is considered the main drawback of SMG as it necessarily poses restrictions on sensitivity.

One of the biggest challenges of current antibiotic resistance research remains pinpointing the bacterial hosts of ARG in complex environmental communities. This issue is nowadays tackled by two complementary approaches. On the one hand, molecular methods are being developed to actually codetect ARG and phylogenetic markers in single cells via sophisticated PCR protocols,[19,20] single-cell sequencing[21] or the sequencing

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of metagenomic DNA that underwent cross-linking prior to extraction.[22,23] On the other hand, there are attempts to link resistance and phylogenetic information exclusively in the computational domain. This includes the assembly of large contigs from fragmented metagenomic DNA[24,25] and the study of empirical associations between the abundances of ARG and phylogenetic markers.[16,26,27]

With the assembly based approaches, reconstructed DNA fragments (contigs) are scanned for physical co-occurrences of ARG and phylogenetic markers on one and the same fragment such that, ideally, ARG−host relations can be deduced from individual samples. By contrast, the statistical approach necessarily compares multiple samples of different community composition to identify associations between the abundance of ARG and taxonomic markers. For the latter approach, ARG and taxonomic markers do not need to reside on identical DNA fragments. Considering that nonassembled fragments obtained from short-read sequencing typically comprise about 150−300 bp only, such co-occurrences actually represent very rare exceptions.

Experience with the different approaches to host identification was recently compiled in a review paper.[28] Of the 21 case studies considered therein, the majority addressed microbiomes of wastewater or waste originating from livestock farming. Cultivation-independent studies on ARG−host associations in freshwater systems, however, are still scarce,[16,29−31] with information being largely confined to eastern Asia.

The aim of the present study is to shed light on ARG−host associations in freshwater biofilms of a European river receiving treated wastewater from multiple plant effluents.

Here, we chose the statistical approach which compares ARG abundances in nonassembled metagenomic DNA across samples of variable community composition. In comparison to metagenome assembly, this approach is computationally cheap, and it consistently covers both chromosomal and plasmid-borne ARG. However, empirical ARG−host associations are prone to spurious correlations[28] arising from hidden effects which simultaneously control resistome and community. The exposure of sampling sites to differing loads of treated wastewater is a typical example of such an effect. Consequently, we advanced the statistical inference of ARG−hosts relations from nonassembled metagenomic DNA by implementing a simple but novel model comparison technique. The latter accounts for simultaneous effects of wastewater disposal on resistomes and bacterial community composition and thus suppresses a major cause of spurious ARG−host relationships. The actual exposure of sampling sites to treated wastewater was assessed through the quantification of crAssphage sequences as proposed recently.[32] To our knowledge, this study is the first of its kind addressing ARG−host relations in a river of central Europe. The outcomes are discussed in light of existing knowledge on the distribution of ARG across taxonomically defined bacterial groups.

MATERIALS AND METHODS

Study Site and Sampling. Samples were taken from the bottom sediment of a German mountain stream and its tributaries (Lockwitzbach River; Figure 1) in September 2017 and July 2018. The spatial distribution of sampling sites was chosen to reflect a gradient of anthropogenic pollution arising from the disposal of municipal wastewater. The latter is discharged into the river at six treatment plants (WWTP) with

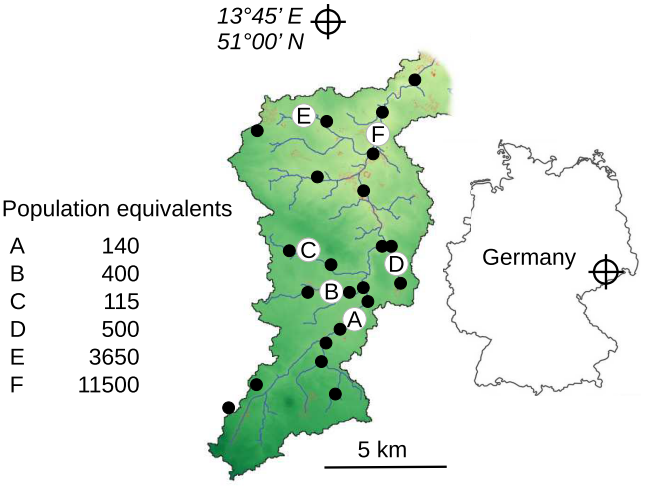

Figure 1. Study catchment with sampling points (filled dots) and wastewater treatment plants (capital letters). Population equivalents reflect nominal treatment capacities reported by the environmental agency of Saxony (LfULG).

capacities ranging from about 100 to 12 000 population equivalents (Figure 1).

The sample material was obtained by pushing a transparent plastic tube vertically into the river bed and was representative of the top 5 cm of bottom sediments. The raw material mainly consisted of sand and silt mixed with varying proportions of gravel. Biofilm was growing on the surface of those particles, and a significant part of it was detached for DNA extraction (details below). Total organic matter accounted for approximately 2−5% of dry weight.[33] For each site, the analyzed sample consisted of two pooled and thoroughly homogenized subsamples to accommodate for small-scale spatial heterogeneity. For reference purposes, effluent water (1 L per sample) was directly collected from the discharge pipe of the easily accessible treatment plant "F" (Figure 1). Glass bottles were used for intermediate storage of sediment material and effluent water at 4 °C before DNA extraction within 12 h after sampling. All glassware was pretreated with sodium hypochlorite to prevent a contamination of samples with foreign traces of DNA.

DNA Extraction and Sequencing. DNA was obtained from both liquid and sediment samples by means of a common extraction protocol. This was to avoid potential bias arising from the use of different protocols on subsets of samples. Consequently, sediment samples were preconditioned by rigorous shaking (450 rpm, 20 min) and the addition of sterile 0.1% sodiumdiphosphate ($Na_4P_2O_7$) solution (20 mL per 100 g wet weight). According to experience,[33] this detaches a significant proportion of bacteria from particle surfaces such that cells residing in the liquid phase are clearly representative of formerly attached biofilm. Liquid-phase material from preconditioned sediment (10 mL per sample) and WWTP effluent water (300 mL per sample) were filtered through 0.2 μm polycarbonate membranes (Whatman, Maidstone, Great Britain). DNA was extracted from filter residues by means of the PowerWaterKit (Qiagen, Hilden, Germany).

The obtained amounts of DNA ranged between 10 and 15 ng mL^{-1} for WWTP effluent water and between 50 and 150 ng DNA mL^{-1} for the sediment. The extracted DNA was stored at −20 °C until sequencing. All samples were shotgun-sequenced

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Environ. Sci. Technol. 2022, 56, 14913−14922

on a HiSeq device (2 × 150 bp paired end) by Eurofins Genomics Europe Sequencing GmbH, Konstanz, Germany.

Bioinformatics. We employed `Trim Galore`[34] to simultaneously remove adapter sequences, barcodes, and low quality information. Specifically, a Phread score of 28 (base call accuracy of 99.85%) was requested. Reads with a length of less than 100 bp after adapter cutting and quality filtering were discarded. Paired end reads were merged with the `pandaseq` assembler[35] using its default settings. The merged sequences were aligned to the `resfinder` database of acquired ARG,[36] version January 2020, available from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) by means of `blastn` (http://blast.ncbi.nlm.nih.gov). Only high quality alignments were retained by requesting a maximum e-value of 1×10^{-20}, a minimum match length of 80 bp, and a sequence identity greater or equal to 95%. A particular read often aligned well with multiple related ARG or ARG variants. Those cases were treated as a single hit and only the longest common name information was retained such that, for example, *tet(M)* and *tet(O)* collapsed into just *tet*. If the gene family could not be identified with certainty, the respective hits were dropped entirely.

The merged reads were further scanned for signatures of 16S rRNA genes with `metaxa2`[37] to identify the taxonomic composition of the bacterial community and to normalize resistance gene counts. Finally, the merged reads were scanned for crAssphage signatures as an indicator of human fecal pollution[32] using the NCBI reference sequence NC_024711.1[38] cut into chunks of 500 bp. Only alignments with e-values $\leq 1 \times 10^{-20}$ and ≥95% identity within a minimum of 80 bp were considered as hits. Basic properties of the samples are summarized in Table 1.

Table 1. Basic Properties of the Analyzed Material[a]

sample material	samples	average number of merged sequences per sample	16S rRNA gene copies per sample (average)
river biofilm	62	$2.5 \times 10^{+7}$	$3.1 \times 10^{+4}$
WWTP effluent	8	$2.2 \times 10^{+7}$	$2.5 \times 10^{+4}$

[a]The number of sequences was counted after merging forward and reverse reads; the average sequence length was 260 bp.

Data Analysis. The strict quality criteria applied in sequence processing and alignment were complemented by additional filter criteria. The latter guard against the misinterpretation of sequencing errors and they help to prevent frequent type II errors merely attributable to very small sample sizes. In particular, we discarded all ARG from analysis which did not reach a threshold frequency of 10 copies in at least one of the samples. Similarly, taxonomically defined bacterial groups were only considered if at least 50 copies of the respective 16S rRNA gene were detected in any of the samples. The number of crAssphage copies per sample ranged between 0 and 166, and the respective information was used without filtering.

Theoretically, the reliability of ARG detection could be compromised by a high degree of redundancy in the sense that most of the reads yielding a hit align to exactly the same short subsequence within a longer ARG fragment. Consequently, we analyzed the subject coverage for every ARG and each sample using an indicator α (eq 1; $0 < \alpha \leq 1$).

$$\alpha = \frac{\mathrm{CS}}{\min(\mathrm{TS}, C_{\max})} \quad (1)$$

where TS represents the total number of nucleotides in the subject sequence (length of gene fragment in resfinder database) and CS is the number of nucleotides of the subject sequence actually covered by hits. Finally, $C_{\max}$ represents the upper limit of *CS* for a theoretical situation where all hits are fully independent without any overlaps. Consequently, $\alpha = 1$ signals optimum conditions with all hits are nonredundant in the sense that they cover independent regions of the subject sequence. Lower values indicate partial redundancy, that is, overlaps between hits in terms of the covered nucleotides of the subject. For our data set, the "global" value of α aggregated over all samples and resistance genes was 0.86. Hence, only a minor fraction (≈15%) of the alignments counted as hits was actually subject to redundancy. At the level of individual genes, the values of α ranged between 0.77 (gene *ant*) and 0.91 (*qnrS*) which was considered acceptable.

To facilitate a comparison of ARG prevalence across samples, the number of ARG copies was generally divided by the corresponding number of 16S rRNA gene copies to yield relative abundances.[39] The number of crAssphage copies found in a sample was normalized to the number of scanned reads reflecting the total amount of analyzed DNA. Models relating the abundance of ARG to the abundance of bacterial groups were fitted using raw gene counts to avoid artificial skill resulting from common normalization.[40]

Data analysis was conducted in R version 4.1.[41] Empirical associations or differences between sample groups were tested for significance with nonparametric methods exclusively, namely "cor.test(method = "spearman")" and "wilcox.test()". Standard linear models were fitted with "lm()" and likelihood ratio tests were performed with "lrtest()". Sorting of matrix rows by similarity was performed by hierarchical cluster analysis ("hclust()", "cutree()"). In the context of multiple hypothesis testing, p values were adjusted with "p.adjust(method = "BH")" to control the false discovery rate.[42]

RESULTS AND DISCUSSION

General Properties of Samples. The average number of reads per sediment sample was 2.5×10^7 with only moderate variation (range: 1.1×10^7–4.5×10^7). At the same time, the samples did not vary substantially in terms of diversity based on the number of unique bacterial orders detected. In a rarefaction plot, all samples fell into the region where the curve levels off suggesting that diversity was adequately represented by the amount of sequenced material (Figure 2). No indication was found for a correlation between bacterial diversity and the amount of crAssphage signatures contained in samples (Spearman's $\rho = 0.07$, $p = 0.4$).

Exposure of Sampling Sites to Treated Wastewater. Wastewater effluents potentially affect both the resistome and the composition of bacterial communities in receiving waters. In statistical analyses targeted at the identification of ARG host, those simultaneous effects must be taken into account to avoid spurious correlations. However, the pollution status of a particular sampling site with regard to treated wastewater is difficult to quantify. This is due to uncertainty in estimated effluent rates, dilution effects, as well as possible in-stream retention all of which are subject to temporal variability. Recently, it has been suggested to employ the signature of crAssphage, a bacteriophage associated with the human gut

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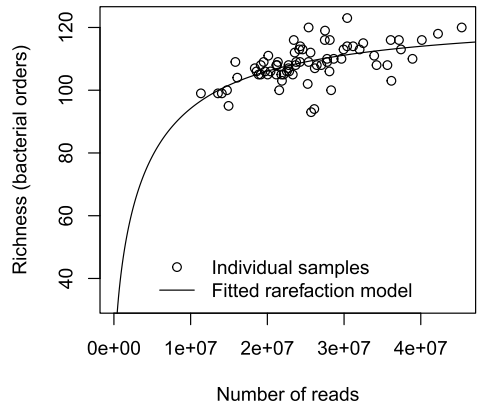

Figure 2. Number of bacterial orders detected in river biofilm in relation to sample size (number of reads). The structure of the fitted theoretical rarefaction model was adopted from Hess et al. 2019.[39]

flora[38] as a tracer for fecal pollution.[32] To verify the applicability of this approach, sampling sites were first grouped into ordinal classes according to the suspected exposure to wastewater: "None"' for sites upstream of any WWTP, "High" for sites immediately downstream of WWTP where effluent and river have just mixed, and "Moderate" for sites in greater distance to upstream plants (≥2 km) where impacts of wastewater have potentially been attenuated by in-stream retention and dilution already. Then, all samples were scanned for crAssphage signatures and relative abundances were compared across the ordinal exposure classes (Figure 3). In

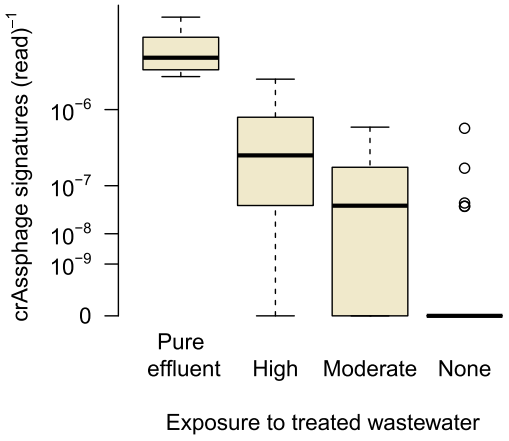

Figure 3. Relative abundance of crAssphage signatures in pure WWTP effluent (very left) and river biofilms with different exposure to treated wastewater. Differences between adjacent groups are significant with all $p < 0.02$ (one-sided Wilcoxon rank sum test).

fact, the number of crAssphage sequences per read differed significantly between sample classes with a continuous increase from unspoiled to highly exposed sites. In accordance with expectation, crAssphage abundance was highest in pure effluent water.

While the majority of samples collected upstream of any WWTP was free of crAssphage signatures, elevated signals were found at some sampling points (outliers in the rightmost box of Figure 3). The respective samples do not stand out from the rest, neither in sequencing depth (number of reads) nor in terms of bacterial diversity. Thus, very likely, these outliers indicate cases of illegal wastewater disposal. However, they could also reflect a lack of specificity since crAssphage signatures have recently been detected in feces of nonhuman origin.[43]

In conclusion, the number of crAssphage signatures per read appears to be a reasonable indicator for the exposure of sampling sites to treated wastewater. Since crAssphage information is continuous and quantitative, it was used as a proxy for wastewater exposure in all downstream statistics.

Impact of Wastewater on Bacterial Community Composition. Understanding the possible impact of wastewater disposal on the composition of in-river bacterial communities is necessary to properly interpret resistome information. In total, 71 bacterial orders were detected based on the chosen filter criteria. For about 20% of those, a positive association between their relative abundance and the exposure of the sampling sites to treated wastewater was established (Table 2). As expected, negative associations were observed as well, reflecting a linkage of certain bacterial groups with primary environmental habitats.

Table 2. Bacterial Orders Whose Relative Abundance in Riverbed Biofilms Was Positively Associated with crAssphage Contamination As an Indicator for Fecal Pollution

phylum	order	Spearman's ρ	adj. p-value
Bacteroidetes	Flavobacteriales	0.51	7.4×10^{-05} ***
Chlamydiae	Chlamydiales	0.51	6.6×10^{-05} ***
Chloroflexi	Chloroflexales	0.28	0.039 *
Cyanobacteria	Subsection I	0.46	0.00033 ***
	Subsection II	0.43	9×10^{-04} ***
	Subsection III	0.35	0.0078 **
	Subsection V	0.35	0.0068 **
Firmicutes	Lactobacillales	0.53	6.1×10^{-05} ***
Fusobacteria	Fusobacteriales	0.49	0.00014 ***
Proteobacteria	Bdellovibrionales	0.47	0.00024 ***
	Legionellales	0.45	0.00053 ***
	Pseudomonadales	0.37	0.0053 **
	Rickettsiales	0.66	3×10^{-08} ***
Tenericutes	Mycoplasmatales	0.38	0.0038 **

The bacterial groups listed in Table 2 form a heterogeneous set. The phyla Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria, for example, are known to make up most of the human gut microbiome[44,45] and a positive association is thus in agreement with expectation. Bacteroidetes and Proteobacteria have also been shown to majorly contribute to the composition of activated sludge microbiomes.[46]

By contrast, the correlation of crAssphage contamination with the abundance of Cyanobacteria is likely attributed to eutrophication caused by incomplete removal of nutrients in sewage treatment.[47] These two examples illustrate how wastewater disposal can potentially shape river microbiomes via mechanisms of selection and invasion. The latter term, however, must be used with care since, depending on bacterial adaptive capabilities, we have to expect all forms of invasion ranging from only transient presence to the persistent integration into freshwater communities.

Although the observed relations between community composition and crAssphage abundance are plausible, the analyses are potentially compromised by the high level of aggregation. In particular, positive associations may remain unidentified due to the simultaneous occurrence of positive

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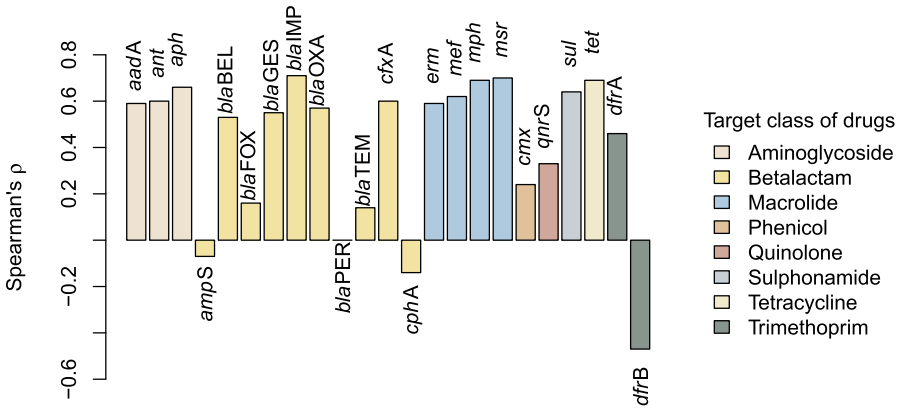

Figure 4. Association of the relative abundance of ARG with the relative abundance of crAssphage signatures. All cases where $|\rho| \geq 0.3$ meet the significance criterion of $p < 0.001$ after correction for multiple testing.

and negative association between crAssphage and taxonomic units at lower phylogenetic levels. For example, if "A" and "B" represent two bacterial families, a positive link between crAssphage and "A" may be "canceled out" by a negative link with "B" when the analysis is performed at order level.

Association of Wastewater Exposure with Antibiotic Resistance Genes. Relative ARG abundance was positively correlated with the exposure of sampling sites to treated wastewater for most of the genes (Figure 4, Supporting Information Table S1). This was especially true for genes conferring resistance to aminoglycosides, macrolides, sulfonamide, or tetracyclines. By contrast, there was no consistent pattern for genes providing resistance to beta-lactams or trimethoprim.

In the case of gene families encoding for beta-lactamases, 5 out of 10 showed a positive association with crAssphage while the remaining five appeared to be unrelated. Between these two groups, no differences were found, neither with regard to the enzyme class nor in terms of the target spectrum of drugs. Specifically, genes coding for class A, B, and D beta-lactamases were present in both groups. Likewise, resistance to carbapenems, cephalosporins, cephamycins, monobactams, penams, or penems was not uniquely linked to either group, and most of the gene families—in both groups—are known to be effective against multiple beta-lactam antibiotics according to CARD records.[48] Heterogeneous trends with regard to the occurrence of distinct beta-lactam resistance genes were also reported for other environments. For example, *bla*TEM genes were found to be ubiquitous in soil microbiomes, whereas the abundances of *bla*OXA or *bla*CTX correlated with anthropogenic impacts.[49,50]

Of particular interest is the result for the two variants of dihydrofolate reductase genes (*dfr*) conferring trimethoprim resistance. While the relative abundance of type I resistance (*dfr*A) was significantly increased in samples contaminated with crAssphage signatures, the opposite was true for type II (*dfr*B). Strikingly, *dfr*B was detected in the majority of riverbed biofilms, whereas it was not detected in any sample of treatment plant effluent which is unlikely to be by chance ($p = 0.023$; Fisher's exact test).

On the one hand, this could point toward an environmental origin of the *dfr*B genes. On the other hand, genes of the *dfr*B family have already been detected in genomes of various clinically relevant strains[51] with clear evidence for mobility.[52] Hence, the detection of *dfr*B in environmental samples could be a result of resistance dissemination predominantly from nonpoint sources. In Germany, combined trimethoprim and sulfonamide treatment is not restricted to human infections but it is also a legal medication in a veterinary context, for example, for cattle, pigs, and pets. Within the catchment, seven larger facilities of commercial livestock farming were identified from aerial images and on-site inspection which could potentially serve as hot-spots of diffuse resistance dissemination. However, a statistical comparison of ARG abundances observed up- and downstream of any of those facilities did not indicate significant effects, irrespective of the gene family (Wilcoxon test; all adj. *p*-values >0.2).

Resistome data from sites located upstream of any treatment plant effluent underwent a separate analysis to better understand the outlier samples depicted as dots in Figure 3. No statistically significant difference in the relative abundance of ARG was found when comparing samples with and without elevated levels of crAssphage signatures. One straightforward conclusion could be that the crAssphage signal was misleading in the sense that it reflected some local contamination with feces of nonhuman origin. However, another plausible interpretation would be that elevated crAssphase levels indeed originated from illegal disposal of wastewater but the contributing population was very small and so was the likelihood of ARG dissemination from actually colonized individuals.

Association of ARG Abundance with Taxonomic Groups. In this work, we followed the statistics-based approach toward the problem of ARG−host identification from metagenomic information. Considering the study design along a gradient of pollution (Figure 1), mere correlations between the abundance of ARG and taxonomic markers are likely to yield spurious outcomes. This is due to the fact that both the abundance of ARG and bacterial groups are often simultaneously affected by wastewater effluents (Table 2, Figure 4). Consequently, we refrained from examining correlations but followed a model comparison approach. Specifically, we fitted linear models to predict the number of ARG copies in a sample, y, using two predictors: the presence of crAssphage signatures, x_1, and the number of 16S rRNA gene copies associated with a particular taxonomic group of bacteria, x_2 (eqs 2 and 3). To infer the possible value of bacterial abundance information, the two-predictor model (eq 3) was compared to the single-predictor version (eq 2) by means of a likelihood ratio test. Models with negative

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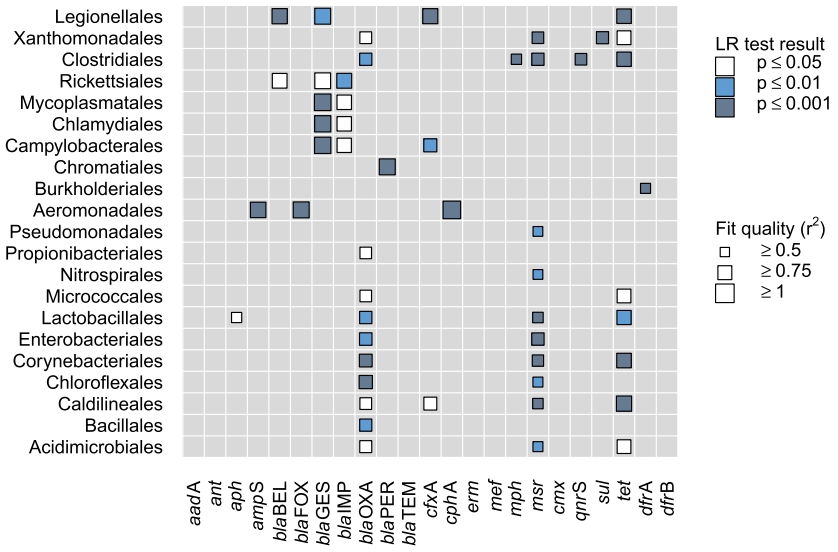

Figure 5. Performance of two-predictor models (eq 3) in relation to the single-predictor benchmarks (eq 2). Colors encode *p*-values of a likelihood ratio (LR) test with the null hypothesis being that community information does not contribute to predictive power; *p*-values were adjusted column-wise to account for the fact that all bacterial orders were considered as candidate predictors. Symbol size depicts the fraction of variance explained by eq 3; cases with $r^2 < 0.5$ were suppressed for clarity. Row order was determined by cluster analysis.

coefficients a_2 were generally discarded since inverse relationship between ARG and bacterial abundances are not of interest in the context of host identification.

$$y = a_1 \cdot x_1 + \epsilon \quad (2)$$

$$y = a_1 \cdot x_1 + a_2 \cdot x_2 + \epsilon \quad (3)$$

The model comparison approach disclosed a heterogeneous pattern (Figure 5).

For the majority of ARG, the incorporation of information on bacterial community composition allowed for an improved estimation of gene abundances. However, results differ substantially between particular ARG families. In the case of beta-lactam resistance genes like *amp*S, *bla*FOX, *bla*PER, or *cph*A, for example, only a single bacterial order contributed significantly to model performance. Thus, the respective orders are likely to play an actual role as host of the respective ARG.

By contrast, genes like *bla*GES, *bla*IMP, *bla*OXA, *msr*, or *tet* were found to be linked with numerous bacterial orders leaving more room for speculation. While all these orders could potentially serve as hosts, such outcomes are rather likely to reflect collinearity. The latter can arise from associations between bacterial community members due to, for example, common environmental preferences or even metabolic dependencies. Nevertheless, even those results are not entirely inconclusive since Figure 5 still depicts characteristic patterns. For example, the genes *bla*GES and *bla*IMP conferring resistance to carbapenems were found to be linked with a different set of bacterial orders than, for example, ARG of the *bla*OXA or *tet* group.

Finally, Figure 5 also highlights a few ARG families whose relative abundance was not significantly associated with any bacterial order (e.g., *aad*A, *ant*, *erm*). Such an outcome would be expected for ARG that are widely spread across taxonomic groups possibly facilitated through horizontal transfer of plasmids with a broad host range. In fact, genes like *aad*A or *erm* have been detected in chromosomal and plasmid DNA of numerous bacterial hosts according to current CARD[48] records, including Gram-positive and negative species. Furthermore, the particular role of horizontal transfer for the dissemination of these genes has been highlighted earlier.[53,54]

Several of the bacterial orders highlighted in Figure 5 harbor potentially pathogenic species with drug resistance being clinically relevant. This includes, for example, Aeromonadales, Clostridiales, or Enterobacteriales, while some of the other bacterial groups have not been in the focus of drug resistance research so far. Unfortunately, a validation of the empirically identified relationships suggested by Figure 5 is not easily accomplished. On the one hand, case studies on ARG−host relationships in surface waters are not only limited in number but also with regard to geographical coverage.[16,29−31] On the other hand, whole genome-based information on ARG prevalence found in repositories like CARD[48] is potentially strongly biased toward culturable bacteria of clinical relevance.

Nevertheless, some of the ARG−host relations proposed here are strongly supported by previous studies on the phylogeny of resistance genes.[55,56] This is especially so for ARG that were positively linked to the order Aeromonadales. For instance, in agreement with Figure 5, the fish pathogen *Aeromonas allosaccharophila* was recently identified as the original host of *bla*FOX genes.[57] Similarly, the *cph*A gene was previously found to be linked with the host *Aeromonas hydrophila*.[58]

The association of *bla*PER beta-lactamases with the bacterial order Chromatiales implied by Figure 5 represents another case supported by external evaluation. Only recently, it was suggested that *bla*PER genes were originally acquired by the genus *Pararheinheimera*, a member of Chromatiales, long before the era of antibiotics[59] with the occurrence in human pathogens being a result of later horizontal transmission.

At a higher level, the candidate associations proposed here support the outcome of previous research on ARG−host associations[28] according to which the majority of hosts falls into the groups of Proteobacteria and Firmicutes. These two

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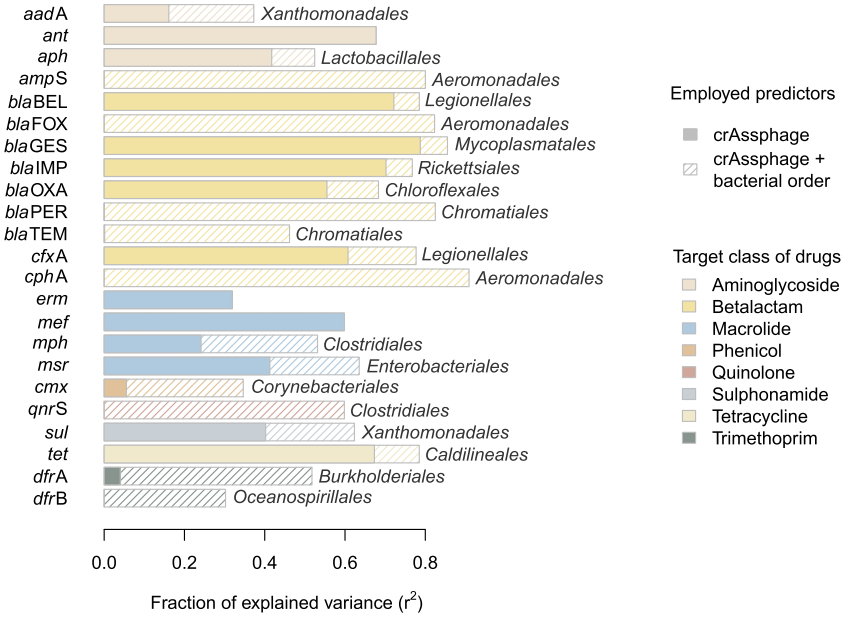

Figure 6. Performance of linear models predicting the relative abundance of ARG families from crAssphage abundance (eq 2; solid bars) or crAssphage abundance combined with information on bacterial community composition (eq 3; total bar length). For each ARG family, the indicated bacterial order is the one that allowed for the best fit of eq 3 and shaded parts represent the corresponding improvement of the model. If no order name is given, community information had no added value whatsoever.

phyla contribute the majority of bacterial orders depicted in Figure 5.

The cases of *bla*FOX, *bla*PER, and *cph*A clearly illustrate that the statistical inference of ARG hosts from nonassembled metagenomic data is actually feasible. However, challenges remain with regard to several aspects. First of all, it seems necessary to apply the approach to even larger data sets exhibiting considerable variation in the composition of bacterial communities. On the one hand, this would allow for an improved detection of false positives by, for example, cross-validation methods. On the other hand, it may help to further disentangle some of the clusters depicted in Figure 5 where a particular set of ARG was associated with multiple bacterial groups and host relations thus remain inconclusive.

Second, it seems necessary to further increase resolution, especially of the taxonomic data as pointed out recently.[28] This is due to considerable diversity at higher taxonomic levels like orders or phyla which may obscure ARG−host relations manifested at genera or species level. In this respect, progress could be made through sequencing technologies yielding longer reads.[60]

Third, the statistics-based approach to the identification of ARG can certainly profit from independent validation data produced with complementary techniques like, for example, metagenome assembly[61] or Hi-C sequencing.[23] A sole validation against information on ARG prevalence observed in isolates (many of which having a clinical background) can hardly suffice. Even worse, it could result in the false rejection of identified ARG−host relationships in environmental systems for the reason for apparent implausibility.

To possibly validate the ARG−host associations reported here, we applied the memory-efficient MEGAHIT software[62] to assemble six of the metagenomes. About $^{1}/_{5}$ of the ARG and $^{1}/_{10}$ of the 16S rRNA-based taxonomic markers detected on the original reads were recovered from contigs. However, among the 8×10^{6} generated contigs there were only three instances with simultaneous information on resistance and host. All three cases were related to the macrolide resistance gene *msr*E with Comamonadaceae (order Burkholderiales; 2×) and Microoscillaceae (order Cytophagales; 1×) being reported as the candidate hosts. This amount of information is clearly insufficient for serious validation. In our case, the major limitation appeared to be the insufficient length of the assembled contigs. While a few long sequences of up to about 900 kbp were obtained, median contig lengths ranged between 590 and 800 bp only and the codetection of ARG and specific 16S rRNA information was thus unlikely. Moreover, one needs to keep in mind that assembly based strategies are not suited to the identification of ARG host when the resistance is plasmid-borne. In fact, the full consideration of plasmid-borne ARG is one of the major advantages of the statistical approach to host identification.

A successful identification of ARG−host relations by the statistical approach requires a number of conditions to be met. First of all, multiple metagenomic samples must be analyzed each contributing millions of reads. A high number of reads per sample is the key to the detection and quantification of less abundant bacterial groups and ARG. Likewise, statistical significance of empirical ARG−host relationships is more likely to be established the more individual samples are available. However, a high number of deeply sequenced samples alone does not guarantee success. Rather, it is crucial that individual samples differ with regard to community composition. Without variance in community composition, the statistics-based inference of ARG−host relations would clearly be infeasible.

Finally, it needs to be stressed that any ARG−host relations proposed by statistical analyses should be treated with the usual caution. First, false positives cannot be avoided entirely and a small proportion of the identified associations may thus reflect coincidence. This is especially so when the number of multiple tests becomes large, for example, in response to a fine-

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Environ. Sci. Technol. 2022, 56, 14913−14922

grained decomposition of the bacterial community. To guard against false positives, obtained *p*-values should undergo rigorous adjustment as in the preparation of Figure 5. Second but more important, candidate bacterial groups must not be regarded as the "exclusive hosts". While they potentially play an outstanding role, many ARG families have invaded multiple branches of the phylogenetic tree by horizontal transfer and certainly persist in multiple bacterial orders or phyla.[63,64]

To better illustrate the possible contribution of taxonomic information to the prediction of ARG prevalence, the matrix from Figure 5 was filtered to those bacterial orders that allowed for the best fit of eq 3. Furthermore, the fraction of explained variance was decomposed so as to highlight the contribution of community information in relation to crAssphage information (Figure 6). For more than $^3/_4$ of the gene families, crAssphage data combined with information on a single bacterial order explained over 50% of the observed variance in ARG abundance. For genes conferring resistance to beta-lactam antibiotics, up to about 80% of the variance were explained.

Synthesis. We found strong evidence for an impact of wastewater effluents on both the taxonomic composition of river bacterial biofilms and their enrichment with ARG. With regard to the latter, the strongest effects were observed for gene families conferring a reduced susceptibility to aminoglycosides, macrolides, tetracyclines, and selected beta-lactam antibiotics including carbapenems. However, the abundance of several other ARG families coding for beta-lactamases (e.g., *bla*TEM, *ampS*) or type II trimethoprim resistance (*dfr*B) was apparently unrelated to wastewater disposal. Hence, the respective genes are either naturally present in river microbiomes independent of human impacts or they are linked with anthropogenic nonpoint emissions.

The statistics-based approach to the identification of ARG hosts yielded promising results. In several cases, the relative abundance of a particular ARG proved to be significantly associated with the occurrence of just one or two specific bacterial orders. For several ARG families mediating beta-lactam resistance (*bla*FOX, *bla*PER, *cph*A) the proposed host relationships strongly support recent external evidence rooted in evolutionary analyses. However, even cases where a particular ARG is found to be associated with multiple bacterial groups (or no group at all) are not without relevance. In fact, those cases may reflect an efficient horizontal transfer of antibiotic resistance through highly mobile genetic elements and call for further examination. Overall, community composition proved to be a valuable input to statistical resistome models. Specifically, for 20 out of 23 resistance gene families, information on the abundance of particular bacterial orders significantly improved the performance of linear models predicting ARG abundance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c00370.

Table S1: Relative abundance of antibiotic resistance genes (ARG copies/16S rRNA gene copies) in river biofilms sampled downstream of wastewater effluents ("highly exposed") in comparison to values observed upstream of any treatment plant ("not exposed"). Reported *p*-values refer to a Wilcoxon rank sum test and underwent adjustment for multiple testing. Stars indicated significance according to the usual convention (PDF)

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Notes

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Environ. Sci. Technol. 2022, 56, 14913−14922