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Abstract

There is concern that antibiotics in the environment can select for and enrich bacteria carrying acquired antibiotic resistance genes, thus increasing the potential of those genes to emerge in a clinical context. A critical question for understanding and managing such risks is what levels of antibiotics are needed to select for resistance in complex bacterial communities. Here, we address this question by examining the phenotypic and genotypic profiles of aquatic communities exposed to ciprofloxacin, also evaluating the within-species selection of resistant *E. coli* in complex communities. The taxonomic composition was significantly altered at ciprofloxacin exposure concentrations down to 1 µg/L. Shotgun metagenomic analysis indicated that mobile quinolone resistance determinants (*qnrD*, *qnrS* and *qnrB*) were enriched

as a direct consequence of ciprofloxacin exposure from 1 µg/L or higher. Only at 5-10 µg/L resistant *E.coli* increased relative to their sensitive counterparts. These resistant *E. coli* predominantly harbored non-transferrable, chromosomal triple mutations (*gyrA* S83L, D87N and *parC* S80I), which confer high-level resistance. In a controlled experimental setup such as this, we interpret effects on taxonomic composition and enrichment of mobile quinolone resistance genes as relevant indicators of risk. Hence, the lowest observed effect concentration for resistance selection in complex communities by ciprofloxacin was 1 µg/L and the corresponding no observed effect concentration 0.1 µg/L. These findings can be used to define and implement discharge or surface water limits to reduce risks for selection of antibiotic resistance in the environment.

Keywords Antibiotic resistance, NOEC, LOEC, Environmental emission limits

Abbreviations

ARG, Antibiotic resistance gene; LOEC, Lowest effect concentration; MIC, Minimal inhibitory concentration; MSC, Minimal selective concentration; NOEC, No effect concentration; QRDR, Quinolone resistance-determining region; WWTP, Wastewater treatment plant; OGLM, Overdispersed poisson linear model

1. Introduction

The rapid emergence and dissemination of antibiotic resistant bacterial pathogens is one of the most pressing threats to public health (WHO, 2014). Antibiotic resistance genes (ARGs) were, however, present in the environment long before antibiotics were introduced as clinical agents (D'Costa *et al.*, 2011). Since then, many ARGs have been acquired and enriched in pathogens under a selection pressure from antibiotics. For example, the quinolone resistance gene *qnrA* is hypothesized to originate from the waterborne species *Shewanella algaeas* and has now spread to clinical isolates of different species within *Enterobacteriaceae* (Corkill *et al.*, 2005, Poirel *et al.*, 2005). Likewise, bacterial species occurring in the environment were identified as origins of the quinolone resistance gene *qnrB* and the carbapenem-hydrolyzing oxacillinase *OXA-181* (Jacoby *et al.*, 2011, Potron *et al.*, 2011). For most ARGs, however we know neither the origin nor the circumstances under which they were transferred to pathogens. Nevertheless, it is highly plausible that man-made antibiotics played and continue to play a critical role in this process.

Selection of antibiotic resistant bacteria can take place not only in or on the bodies of humans and domestic animals given antibiotics, but also in the external environment (Alonso *et al.*, 2001, Martinez, 2008). Wastewater treatment plants (WWTP) have been mentioned as potentially important arenas for the emergence of new forms of resistance in pathogens (Rizzo *et al.*, 2013). WWTPs are characterized by a coexistence of numerous intestinal commensals and pathogens, a wide diversity of environmental bacteria that may act as donors of novel resistance elements, and a mixture of antibiotics, antibacterial biocides and metals that all might contribute to selection of antibiotic resistant strains. We and others argue that the flow of ARGs from the vast environmental reservoir to pathogens must be managed to prolong the utility of antibiotics (Bengtsson-Palme & Larsson, 2015, Martinez *et al.*, 2015). Hence, thorough knowledge regarding selective concentrations in the environment is urgently needed

85 to guide mitigations in order to limit environmental selection of antibiotic resistance
86 (Bengtsson-Palme & Larsson, 2017, Le Page *et al.*, 2017).

87 Fluoroquinolones, including ciprofloxacin, are recognized by the WHO as critically important
88 antibiotics for human medicine (WHO, 2017). The primary targets of fluoroquinolones are the
89 bacterial type II topoisomerase DNA gyrase and topoisomerase IV, which are enzymes
90 involved in the supercoiling of DNA and thus impair DNA replication (Drlica, 1999).
91 Bacterial resistance against fluoroquinolones can arise from several mechanisms, including
92 mutations in the genes encoding the targeted type II topoisomerases, increased drug efflux
93 and transmissible target protection mechanisms (Redgrave *et al.*, 2014). Fluoroquinolones are
94 furthermore characterized by a high degree of persistence in the environment (Kümmerer *et*
95 *al.*, 2000, Golet *et al.*, 2003, Cardoza *et al.*, 2005). Concentrations found range from low ng/L
96 to at the most a few µg/L as a result of excreted drugs from humans and animals (Golet *et al.*,
97 2002, Zuccato *et al.*, 2010, Bengtsson-Palme *et al.*, 2016, Ory *et al.*, 2016). In contrast,
98 exceptionally high levels of ciprofloxacin (mg/L) and other fluoroquinolones have been found
99 in effluent from drug manufacturers and in nearby, industrially polluted environments
100 (Larsson *et al.*, 2007, Fick *et al.*, 2009, Kristiansson *et al.*, 2011, Gothwal & Shashidhar,
101 2017). While it is indisputable that such high levels, way above the clinical breakpoint for
102 most pathogens, will select for resistance, it is unclear what role much lower concentration
103 might play, and hence where and to what extent mitigations are needed.

104 The relationship between the antibiotic concentration and its ability to select for resistance is,
105 accordingly, critical to understand. Gullberg et al. used a test tube-based competition assay to
106 determine the minimal selective concentration (MSC) of ciprofloxacin for *E. coli* (Gullberg *et*
107 *al.*, 2011). The lowest experimentally tested concentration that indicated a growth advantage
108 of the *E. coli* harboring a resistance mutation in *gyrA* (S83L) was 0.23 µg/L. Through
109 extrapolation of growth data over a series of concentrations, they predicted that selection

would potentially occur down to 0.1 µg/L. Consequently, a concentration below this predicted threshold would favor the sensitive variant and would not compensate for the growth impairment caused by the resistance mutation. This defined the MSC. Using a rather similar competition assay, studying the very same resistance mutation in *E.coli*, Liu et al. demonstrated selection for resistance at 3 µg/L, but not at 2 µg/L (Liu *et al.*, 2011). Although it is not entirely clear why the studies differ quantitatively, it has likely to do with the more sensitive cell counting method used by Gullberg et al. (2011).

Notably, both of the above competition studies were performed under optimized laboratory conditions in a nutrient rich environment and with only two isogenic lab strains differing by one mutation competing against each other. Such studies likely give much valuable insights regarding the potential of antibiotics to select for resistant strains in environments characterized by low bacterial complexity and favorable growth conditions. Most microbial ecosystems, however, harbor many species competing for resources and the availability of nutrients varies considerably over space and time, placing quite different demands on a successful variant. Depending on the composition of the community, antibiotics may select for a range of chromosomally encoded resistance mutations as well as mobile genetic elements that may be transferred across strains and species, and a combination of both. Most importantly, selection in communities may occur primarily on the species level, with intrinsically resistant species filling the niches made available, rather than providing growth opportunities for strains with acquired resistance. Such between species selection could increase the abundance of species acting as donors of resistance factors. However, it is not as critical from a clinical risk perspective, as it would not *per se* contribute to the fixation of acquired resistance (Andersson & Hughes, 2011), nor would it generally increase the transmission possibilities of resistant pathogens (Pal *et al.*, 2017). In a two-strain competition system, even costly resistance factors that confer a minor fitness advantage will take over the

whole culture once a critical antibiotic concentration is reached (Andersson & Hughes, 2010). In a more complex community, other genotypes and species are more likely to fill up such a niche. In the environment, most bacteria exist in surface-associated multicellular communities, so-called biofilms. These are known to produce extracellular polysaccharides that on the one hand shape the biofilms architecture, and on the other hand cause a decreased permeability of toxicants, shielding sensitive species (Donlan & Costerton, 2002, Branda *et al.*, 2005). The resistant biofilm population could also degrade an antibiotic and thus protect the more sensitive variants, as it was shown for TEM-1 β-lactamase expression in *E. coli* (Dugatkin *et al.*, 2005, Perlin *et al.*, 2009). Some bacteria are apparently capable of *catabolizing* synthetic fluoroquinolones (Dantas *et al.*, 2008), but the nature of such enzymes is still unknown. So far, only quinolone *modifying* enzymes have been described, such as the aminoglycoside acetyltransferase AAC(6′)-Ib. Taking into account all of these aspects, selective concentrations derived from pairwise competition experiments may not be directly applicable to microbial communities.

Berglund et al. investigated the effect of a mixture of antibiotics, including ciprofloxacin (up to 20 µg/L nominal concentration) and norfloxacin (100 µg/L), on ARGs and integron prevalence using complex microcosms consisting of water and sediment from a Swedish lake (Berglund *et al.*, 2014). Upon dosing the microcosm with antibiotics and following for 100 days, no increased prevalence of *qnrS* or the integrase gene *intI1* could be observed. However, already on the first day, measured concentrations of fluoroquinolones in the mesocosms were reduced between 90 and 95% from nominal concentrations, and they continued to decrease over time. Furthermore, the closed, nutrient poor experimental system used did likely not enable sufficient bacterial growth in order to detect small or moderate selective advantages of resistant bacteria.

To overcome these drawbacks, a continuous flow-through system was established to determine selective properties of antibiotic agents in complex aquatic bacterial biofilms (Lundström *et al.*, 2016). This system enables studying the formation of complex bacterial communities in selection tanks where the only varying variable, the antibiotic concentration, can be controlled throughout the exposure period. By monitoring changes on phenotypic, genotypic and taxonomic levels, the selective potency of ciprofloxacin on a complex community was investigated. Despite the apparent high sensitivity, a limitation with the approach by Lundström et al. (2016) was that within-species selection was not studied specifically. Also, the setup is rather cost and labor-intense which restrict analysis with high throughput.

The aim of this study was to identify the Lowest Observed Effect Concentration (LOEC) of ciprofloxacin in a complex aquatic bacterial community, studying a range of endpoints that inform about risks for selection of resistance. These included taxonomic composition, within-species selection of resistant strains (*E. coli*), chromosomal resistance mutations, as well as transferrable resistance genes. This was studied in biofilms using sewage effluent inoculum and the flow-through system that has been described recently (Lundström *et al.*, 2016), but we also made a brief comparison with a much less elaborate planktonic test tube culture with serial transfer of complex sewage effluent communities.

2. Material and Methods

2.1. Establishing selective concentration for ciprofloxacin resistance in a test tube system

Test tubes containing 5 mL 10x diluted R2A broth (Reasoner & Geldreich, 1985) without starch and magnesium sulfate supplemented with different concentrations of ciprofloxacin were inoculated with 50 µL effluent diluted 100x prior to use. A grab sample of treated sewage effluent (Ryaverket, Gothenburg, Sweden) served as inoculum. In total ca 747,000 persons are connected to the wastewater treatment plant Ryaverket (Videbris, 2017). Together with industries and other activities in the catchment area the total input corresponds to 981,000 person equivalents. The treatment process includes mechanical, chemical, and biological activated sludge treatment with no final disinfection step. The cultures that were inoculated with treated sewage effluent were prepared in triplicate and incubated over night at 25 °C with shaking (160 rpm). After 24 h, cultures were diluted 1:1000 in fresh medium supplemented with the same antibiotic concentration and incubated for additional 24 h. *E. coli* can be differentiated from other coliforms by its blue color on CHROMagar™ ECC media. At time points 0, 24 h and 48 h a small fraction of culture was plated on CHROMagar™ ECC and R2A agar medium with or without ciprofloxacin (2 µg/mL) in triplicate for each dilution. Enumeration on R2A agar was added to allow the study of selection of other bacteria than *E. coli*, but with the price that within-species selection could not easily be determined, as several species, including also intrinsically resistant ones would grow on the R2A. CHROMagar™ ECC plates were incubated 24 h at 37 °C and R2A agar plates at room temperature for 5 days in the dark. CFUs were enumerated using the median of the plate replicates, followed by the median of each technical replicate. Relative resistance was determined by comparing CFU counts of control and ciprofloxacin containing plates.

2.2.Establishing selective concentration for ciprofloxacin resistance in a flow-through system

2.2.1. Flow-through system for biofilm growth

Biofilms were established on glass slides in a flow through aquaria system as previously described (Lundström *et al.*, 2016). Grab samples of treated sewage effluent (Ryaverket, Gothenburg, Sweden) in a 100x saline dilution served as inoculum and was changed daily. Grab samples were collected twice, at day 0 and 5, and stored at 4 °C in the dark to guarantee equal numbers of viable cells in the inoculum throughout the experiment. Each aquarium had 3 different inlets with the following flow rates: inoculum 1 mL/min, nutrient 0.2 mL/min and ciprofloxacin 0.035 mL/min. Nutrient stocks consisted of 10x diluted R2A broth without starch and magnesium sulfate. At the start of the experiment, 2 L of inoculum, nutrient and ciprofloxacin were mixed according to their flow rate proportions and supplied to fill the aquaria. The suspension in the aquaria was mixed with a magnetic stirrer and kept at room temperature. Biofilms were established under different ciprofloxacin end-concentrations (0, 0.1, 1 and 10 μg/L) in triplicate aquaria.

2.2.2. Measurement of ciprofloxacin concentration in selection aquaria

Ciprofloxacin (Sigma-Aldrich, Steinheim, Germany) used for standard preparation was classified as analytical grade (>98%) and $^{13}C3^{15}N$-labelled ciprofloxacin (Cambridge isotopes labs, London, United Kingdom) was used as internal standard. LC/MS grade quality of methanol and acetonitrile were purchased (Lichrosolv - hypergrade, Merck, Darmstadt, Germany) and the purified water was prepared using a Milli-Q Advantage, including an UV radiation source, ultrapure water system (Millipore, Billerica, USA). Formic acid (Sigma-Aldrich, Steinheim, Germany) was used to prepare the 0.1% mobile phases.

A Thermo TSQ Quantum EMR triple quadrupole mass spectrometer was used for all measurements according to the method by Lindberg et al. (Lindberg *et al.*, 2014). In short, 10 mL of water was filtered through a 0.45 µm syringe filter and was acidified using Formic acid and spiked with isotopically labelled internal standards. The samples were then analyzed using an Inline-Solid phase extraction system with an Oasis HLB column connected to a Thermo Heated electrospray triple quadrupole tandem mass spectrometer.

Two MS/MS transitions were used for positive identifications of ciprofloxacin with the criterion that the ratio between the transitions was not allowed to deviate more than +/−30% from the ratio in the calibration standard. Retention times also had to be within +/−2.5% of the retention time in the calibration standard. Carry-over effects were evaluated by injecting standards at 1000 ng/L followed by two mobile phase blanks. Several instrumental and field blanks were included in the analytical runs.

2.2.3. Harvesting of biofilms

After 9 days, clearly visible biofilms were scraped from the glass slides with sterile cell scrapers (Sarstedt, Germany) and collected in 4 mL of saline. For each aquarium, 3 technical replicates consisting of biofilms from 5 glass slides were harvested. The biofilm suspension was disrupted through bead beating using a Tissue Lyser mixer mill (Qiagen, Netherlands) at a frequency of 25.0 Hz with 5 mm stainless steel beads (Qiagen, Netherlands) for 3 min. The disrupted biofilms were then diluted in saline and plated on CHROMagar™ ECC media with or without ciprofloxacin (0.25 or 2 µg/mL) in triplicate for each dilution. After incubating the plates over night at 37 °C, *E. coli* was enumerated for each aquarium by using the median of the plate replicates, followed by the median of each technical replicate. Relative resistance of *E. coli* was determined by comparing control and ciprofloxacin-containing plates.

2.2.4. DNA extraction

After harvesting the biofilms, samples were prepared for storage at -20 ^{0}C until further use by pooling and pelleting 500 µl of each bead-beated technical replicate. DNA was isolated with the DNeasy Blood and Tissue Kit (Qiagen) according to the protocol designed for DNA extraction of Gram-positive bacteria. The integrity of the DNA samples was confirmed on a 1 % agarose gel. The amount and quality of DNA was determined with Qubit (Thermo Fisher Scientific) and Nanodrop ND-1000 (Thermo Scientific).

2.2.5. Amplicon PCR and sequencing

Sequencing of *gyrA* and *parC* amplicons from the DNA isolated from biofilms was performed as described previously with primers shown in Table 1 (Johnning *et al.*, 2015). PCR mixtures contained 1x AmpliTaq® PCR buffer, 25 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each Primer (Table 1), 50 ng DNA template and were conducted in 50 µL working volumes with AmpliTaq® 360 DNA Polymerase (Thermo Fisher Scientific) using the following parameters: denaturation at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C, and finally, extension at 72 °C for 3 min. The PCR products were visualized and separated using a 1.5 % agarose gel containing 1x SYBR® Safe DNA Gel Stain (Invitrogen). The DNA fragment of interest was excised from the gel and purified with the MinElute® Gel Extraction Kit (Qiagen). The purified *gyrA* and *parC* amplicons were pooled for each sample separately and sequenced in one single run. The samples were sequenced at NGI Genomics Production Stockholm using Illumina MiSeq, yielding 2x251 reads with library sizes from 1.09 to 1.73 million reads per sample.

2.2.6. Bioinformatic analysis

The scripts used to implement the methods described below were written in python 2.7. Differential abundance analyses and diversity analyses were conducted in R version 3.2.3.

2.2.6.1. Analysis of amplicon sequences

Adapters and low quality bases were trimmed (-q 25 –length 150) using TrimGalore. To specifically target *E. coli* in the following analyses, the reads were mapped to a database of Enterobacteriaceae *gyrA*/*parC* genes (NCBI nucleotide 'enterobacteriaceae[orgn] AND *gyrA*/*parC*[gene] AND *gyrA*/*parC*[title]') using usearch '–usearch_local' at 98% identity. Though this cut-off is quite conservative and might miss strains that have a greater variability at the *gyrA*/*parC* locus, it minimizes the number of reads falsely assigned to *E.coli*, ensuring specificity of the analysis. The best hit per read was selected by bit-score. If the target sequence was annotated as *E. coli* or *Shigella gyrA*/*parC*, the reads were selected for subsequent analysis (Johnning *et al.*, 2015). The paired reads were merged using vsearch 2.4.3 (--fastq_mergepairs) and dereplicated using 'usearch –derep_fulllength', in order to speed up the following analyses. Chimeric sequences were removed using 'usearch –uchime_denovo' (Edgar, 2016). After these steps, 56.394 (lowest) to 220.187 (highest) reads were left for the subsequent analysis for *gyrA* and 67.624 to 290.391 for *parC*. To identify mutations and their abundances in selected reads of *gyrA*/*parC*, they were aligned read by read to the respective reference (*E. coli* K-12 MG1655, RefSeq locus tags, *gyrA*: b2231; *parC*: b3019;) using MAFFT v7.310 (Katoh *et al.*, 2002) with the options '--addfragments' and '--keeplength', which restricts the length of the alignment to the length of the reference sequence. In the resulting multiple sequence alignment, each amino acid of the aligned read

was compared to the amino acid of the reference sequence at the respective position. If differing amino acids were encountered at the same position, the number of identical reads for the respective variant was added to the total number of mutations in that position. If amino acids were the same, the number of identical reads at the position were counted as 'covered' and unchanged. The proportion of mutations at a position was calculated as number of reads having a mutation at the respective position divided by the number of reads covering the position in total. Co-occurrences of the known *E. coli* resistance mutations *gyrA* S83 and D87 and *parC* S80 and E84 were investigated using the same method. ANOVA and Dunnett's post hoc test were used to determine whether there was a significant difference in mutation abundances between communities exposed to different ciprofloxacin concentrations. Data was log-transformed in order to ensure approximate normality.

2.2.6.2. Metagenomic analysis

The samples were sequenced at NGI Genomics Production Stockholm using Illumina HISeq2500, yielding 2x125bp reads with library sizes ranging from 109 to 190 million reads per sample.

2.2.6.3. Adapter trimming and generation of antibiotic resistance gene count data

Adapters and low quality bases were removed using TrimGalore v0.4.4 (Babraham Institute, 2010, Martin, 2011), with minimum read length set to 60 bases (--length 60) and a quality cutoff of 25 (-q 25). To detect reads derived from known ARGs, the trimmed reads were then mapped to the Resqu resistance gene database (version 1.1,

http://www.1928diagnostics.com/resdb) using usearch v8.0.1445_i86linux64 (Edgar, 2010). In addition, the database was modified to contain recently predicted *qnr* genes (Boulund *et al.*, 2017). To prevent a situation where an effect is masked through reads randomly mapping to several highly similar sequences, the predicted *qnr* genes and *qnr* genes present in Resqu were clustered at 90% similarity, resulting in 25 *qnr* clusters (supplementary data 2 – qnr clusters). After adding the predicted *qnr* genes, the database was deduplicated using usearch '-derep_fullength'. Each sequence was marked as belonging to its respective cluster, and all hits on genes belonging to the same cluster were summed up and treated as belonging to one entry in the database. To minimize the amount of false positives, the '-usearch_global' option was used during mapping, requiring a minimum sequence similarity of 90% over the whole read length (-id 0.9) for a read to be counted as hit. Metaxa2 was used to assign taxonomy to the trimmed reads and determine the number of reads mapping to 16S rRNA in each sample (Bengtsson-Palme *et al.*, 2015). To compare abundances of resistance genes between samples, the counts were normalized by length and 16S rRNA in the sample (Bengtsson-Palme *et al.*, 2017). For further analysis, the raw count files were merged into a matrix containing gene names as rows, sample names, library size and total count of 16S rRNA reads as columns and the respective raw counts as values. For a separate analysis of efflux pumps, entries containing the terms 'efflux' and 'pump' were extracted from Resqu and BacMet, a database containing biocide and metal resistance genes (Pal *et al.*, 2014). From BacMet (version2), entries were only extracted for genes with experimentally confirmed resistance function. Entries from both databases were merged and clustered at 95% identity using uclust '-cluster_smallmem' (-id 0.95), to again avoid masking effects of highly similar sequences during mapping. In total, 145 of 367 different ARGs, 15 of 17 IS*CR*/*intI* genes and 97 of 336 efflux pumps were detected in the samples. After filtering out genes that had an average of

less than 7 reads per sample mapped to them, 51 ARGs, 10 IS*CR*/*intI* genes and 67 efflux pumps remained for further analysis.

2.2.6.4. Differential abundance analysis

To analyze whether resistance genes that *a priori* are known or suspected provide resistance to fluoroquinolones (supplementary data – qnr clusters) were more abundant in communities exposed to different concentrations of ciprofloxacin, an overdispersed Poisson linear model (Jonsson *et al.*, 2016) (OGLM) was used. The model describes the raw count data as a function of ciprofloxacin concentration, using the log transformed 16S rRNA counts as offset, thus accounting for the different amounts of bacterial reads in each sample. The count data obtained from the communities not exposed to ciprofloxacin were used as intercept, representing the number of resistance genes in the absence of ciprofloxacin. Regression analysis was used to identify differentially abundant genes. To control the false positive rate, the Benjamini-Hochberg correction for multiple testing was applied. To determine at which concentrations changes occurred, pairwise comparisons were conducted for all genes between 0 µg/L and every other concentration separately, through comparison of an OGLM containing ciprofloxacin concentration as a covariate with the same OGLM lacking ciprofloxacin concentration as covariate. The resulting one-tailed p-values were corrected for multiple testing as described above.

In addition to the directional analyses of quinolone resistance genes, we also analyzed changes (up or down) in all resistance genes as described in the sections above (two-tailed hypothesis), efflux pumps and on a set of mobile genetic elements (integrases and Insertion Sequence Common Regions; ISCRs (Flach *et al.*, 2017). Finally, the same methodology was

applied to investigate the differential abundance of bacterial taxa at different ciprofloxacin concentrations.

2.2.6.5. Diversity analysis

A matrix similar to the one described for resistance gene counts (2.2.6.3.) was created, containing the 16S rRNA counts of bacterial genera as classified by Metaxa2. To account for the variable library sizes, the matrix was resampled with the lowest 16S rRNA count of all libraries (74234 reads) using the rrarefy function from the vegan 2.4-3 package (Oksanen *et al.*, 2007). Vegan 2.4-3 was also used to determine the number of species per sample (specnum function) and calculate the Shannon diversity (diversity function) of each sample. To investigate whether a relationship existed between the observed diversity and the concentrations of ciprofloxacin, a linear model explaining the diversity indices by the concentrations was generated. Linear models were also created for the richness and evenness per sample explained by the concentrations. All values used in the models were log10 transformed to ensure approximate normality.

2.2.7. Allele specific PCR on E. coli isolates

The mere abundance of single nucleotide polymorphisms on the *gyrA* and *parC* allele of *E. coli* can be determined by amplicon sequencing as described above. This approach generates a highly detailed picture of occurring polymorphisms and their respective prevalence in each sample. However, this set of data lacks valuable insight about combinations of mutations that often occur in *gyrA* and *parC*, simultaneously. Therefore, allele specific PCR was performed additionally on randomly selected *E. coli* isolates with primers detecting the most prominent

combinations of mutations in the quinolone resistance-determining region (QRDR) of *E. coli*. The Web-based Allele-Specific PCR assay designing tool (WASP) was used to design primers shown in Table 1, which allow precise distinction between the wild-type allele and polymorphisms in *gyrA* (S83L, D87N) and *parC* (S80I) (Wangkumhang *et al.*, 2007). *E. coli* isolates obtained from CHROMagar™ ECC Media with 0.25 µg/mL ciprofloxacin were freshly plated on blood agar and directly used as DNA template. The following mixture was prepared with a final concentration of AmpliTaq® PCR buffer 1x, Magnesium chloride 2mM, 0.25 µl AmpliTaq® 360 DNA Polymerase (Thermo Fisher Scientific), 200 µM of each dNTP mix and 1 µM of each Primer. The following PCR parameters were used: initial denaturation at 95 ^{0}C for 3 minutes, followed by 30 cycles of 30 at 95 ^{0}C, elongation at 72 ^{0}C for 45s, annealing for 30 s at 55 ^{0}C, ending with 3 min at 72 ^{0}C. PCR products were verified on a 1 % agarose gel. Specificity of allele specific primers was confirmed by testing 20 randomly chosen *E. coli* isolates with varying MICs with both sets of allele specific primers and, additionally, by sequencing of the nucleotide location of interest. Comparison of both methods yielded 100% identical outcomes. PCR products were purified with the MinElute® Gel Extraction Kit (Qiagen) and sequenced by GATC Biotech (Konstanz, Germany). When no PCR product could be observed with allele specific primers, this indicated the occurrence of a different mutation than the primers were originally designed for. In those cases PCR products were obtained, purified and sequenced with other primers that covered the nucleotide region of interest (Table 1). Hence, rare mutations are reported as well.

2.2.8. Minimal inhibitory concentration of isolated E. coli strains

E. coli isolates of aquaria without ciprofloxacin and with 10 µg/L exposure, grown on 0.25 mg/L CHROMagar™ ECC media, were stored at -80 ^{0}C. To measure the Minimal Inhibitory

Concentration (MIC), isolates were transferred to MH agar (Oxoide AB, UK) and incubated over night at 37 °C. After one single colony was restreaked and grown over night at 37 °C, inoculum was prepared by suspending isolated bacterial colonies in 0.9 % NaCl. The turbidity of the inoculum suspension was adjusted to 0.5 McFarland. An entire MH agar surface was streaked with a sterile cotton swab soaked in the inoculum suspension. The Etest strip with a range of 0.002 to 32 mg/L ciprofloxacin was applied to the dry MHagar surface and the plates were incubated over night at 37 °C.

421 **Table 1** Oligonucleotide sequences for amplification of target regions in *gyrA* and *parC* of *E. coli*

Gene	Direction	Primer sequence (5´ -> 3´)	PCR product size (bp)
Amplicon PCR using community DNA			
*gyrA*_amplicon	Forward	GGTACACCGTCGCGTACTTT	311
	Reverse	CAACGAAATCGACCGTCTCT	
*parC*_amplicon	forward	GCCTTGCGCTACATGAATTT	287
	reverse	ACCATCAACCAGCGGATAAC	
Allele specific PCR on *E. coli* isolates			
*gyrA*_S83L	forward wildtype allele	CCATCCCCATGGTGAC**AC**	102
	forward mutant allele	CCATCCCCATGGTGAC**AT**	
	reverse	AACCGAAGTTACCCTGACCG	
*gyrA*_D87N	forward	CGATGTCGGTCATTGTTGGC	204
	reverse wildtype allele	CCATGCGGACGATCGTG**AC**	
	reverse mutant allele	CCATGCGGACGATCGTG**AT**	
*parC*_S80I	forward	ATTGTGTATGCGATGTCTGA	128
	reverse wildtype allele	ACCATCGCTTCATAACAGGC**AC**	
	reverse mutant allele	ACCATCGCTTCATAACAGGC**AA**	
PCR on *E. coli* isolates to test specificity of allele specific primer			
gyrA	forward	AGGTTAGGAATTTTGGTTGG	497
	reverse	GTAGAGGGATAGCGGTTAG	
parC	forward	TATGCGGTGGAATATCGGT	550
	reverse	GAAGGCTGGCGAATAAGT	

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3. Results

3.1.Establishing selective concentrations for ciprofloxacin resistance in test tubes

In the simplified test tube experiment, concentrations up to 1 µg/L did not significantly change the fractions of resistant *E. coli* or resistant heterotrophic bacteria, whereas the fractions of both increased significantly at 5 µg/L and higher (Figure 1). A drastic reduction in diversity was observed already after 24 h (Supplementary data, Figure S1).

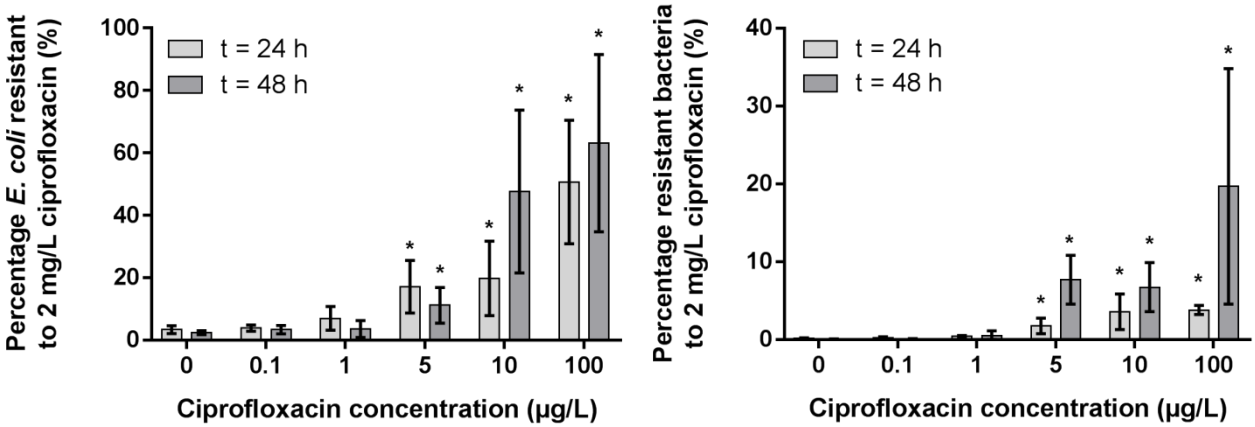

Figure 1 Left: Percent ciprofloxacin resistant *E. coli* grown in complex communities in test tubes with different concentrations of ciprofloxacin (0-100 µg/L) as determined by comparing the number of colony forming units on differential agar medium plates with or without 2 mg/L ciprofloxacin. Right: Percent resistant bacteria grown in complex communities in test tubes as determined by comparing the number of colony forming units on R2A agar medium plates with or without 2 µg/mL ciprofloxacin. Median ± SD values are presented for 3 technical replicates, each consisting of three plating replicates. Two-way ANOVA and Dunnett's post hoc test comparing exposed communities against the control (0 µg/L ciprofloxacin) was performed using log-transformed ratios of resistant to susceptible CFU counts

3.2. Establishing selective concentrations for ciprofloxacin resistance in complex aquatic biofilms

3.2.1. Ciprofloxacin exposure concentration in selection tanks

Measured ciprofloxacin exposure concentrations were close to nominal target concentrations (Table 2). Hence, nominal concentrations are used throughout the manuscript. No ciprofloxacin was detected in control aquaria. The ciprofloxacin concentration detected in the effluent grab sample harvested on day 0 and 5 ranged between 0.015 and 0.02 µg/L. Since these effluents were further diluted 100x prior use, additive effects of ciprofloxacin from the effluents can be neglected.

Table 2 Ciprofloxacin concentrations (µg/L) for each selection aquarium during the exposure response experiment.

Selection aquarium	Target concentration	Measured concentration			Average concentration ± SD
		Day 1	Day 5	Day 9	
T1	0	<LOQ	<LOQ	<LOQ	<LOQ
T2	0	<LOQ	<LQ	<LOQ	
T3	0	<LOQ	<LOQ	<LOQ	
T4	0.1	0.13	0.13	0.1	0.12 ±0.002
T5	0.1	0.16	0.1	0.1	
T6	0.1	0.12	0.13	0.12	
T7	1	0.88	0.99	0.98	1.12 ±0.258
T8	1	1.5	1.27	0.84	
T9	1	n.a.	0.98	1.45	
T10	10	4.7	9.4	7.4	8.84 ±2.691
T11	10	5.7	n.a.	13	
T12	10	10	10	10	

LOQ – Method limit of quantification
n.a. – Sample not available

3.2.2. Phenotypic resistance of *E. coli* in complex aquatic biofilms exposed to ciprofloxacin

A small fraction of the *E. coli*, harvested from the biofilms in selection tanks exposed to up to 1 µg/L, grew on agar plates with 0.25 mg/L or 2 mg/L ciprofloxacin (Figure 2). With the highest exposure concentration of 10 µg/L, the resistant fraction of *E. coli* grown in the complex community drastically and significantly increased to more than 45 % on 0.25 mg/L and 18 % on 2 mg/L ciprofloxacin containing agar plates. A preliminary analysis of heterotrophic bacteria from the biofilms formed under no selection pressure from ciprofloxacin showed that a very high proportion were resistant (41% resistant to 0.25 mg/L and 32% resistant to 2 mg/L ciprofloxacin), likely because of high abundance of intrinsically resistant bacterial species. Such high background resistance reduces the possibility to reliably observe small or moderate changes in resistance patterns. Therefore enumeration on R2A plates was not pursued further.

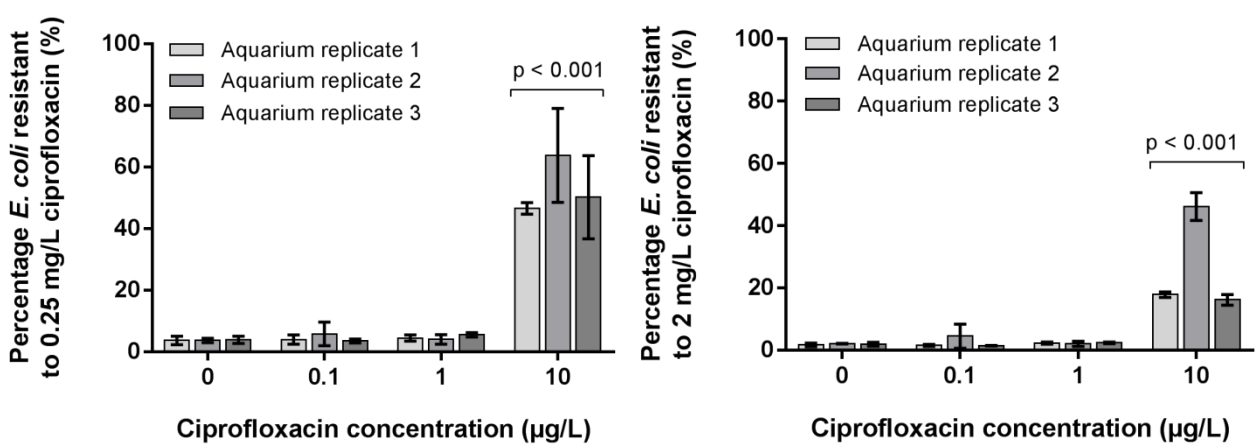

Figure 2 Percent ciprofloxacin resistant *E. coli* cells grown in complex aquatic biofilms with different concentrations of ciprofloxacin (0-100 µg/L) as determined by comparing the number of colony forming units on differential agar medium plates with or without ciprofloxacin (left: selective plates with 0.25 mg/L ciprofloxacin, right: selective plates contained 2 mg/L ciprofloxacin). One-way ANOVA and Dunnett's post hoc test was performed using log-transformed ratios of resistant to susceptible CFU counts to determine statistically significant differences. Significant differences and corresponding p-values compared to control conditions

3.2.3. Genotypic profiling of *E. coli gyrA* and *parC*

3.2.3.1. Amplicon sequencing using community DNA

The most common non-synonymous mutations in *gyrA* were observed in codon S83 (9 – 82 %) and D87 (4 – 41 %) and in *parC* in codon S80 (4 – 54 %) and E84 (3 – 41 %) with increased prevalence of mutations at the highest ciprofloxacin exposure concentration of 10 µg/L (Figure 3, Supplementary data 1 - Table S1 and S2). The most common substitutions in *gyrA* were detected with a relative proportion among the substitutions of 86.3 – 97.1 % for S83L and 72.5 – 95.7 % for D87N in all samples. Approximately half of the reads containing the S83L substitution (37.8 – 49 %) had no D87N substitution in *gyrA* (Figure 4, Supplementary data 1 - Table S3). However, almost all D87N substitutions were identified on alleles that also contained S83L substitutions (96 – 99 %). Non-synonymous variations in *parC* consisted predominantly of S80I with a relative proportion among the substitutions of 85.1 – 98.6 % and of E84V with 72.5 – 95.6 % in all samples. 28 – 44 % of all S80I substitutions had no E84V variation on the same read (Figure 3, Supplements Table S4 and S5). However, E84V substitutions were mostly detected together with S80I substitutions (90 – 96 %) (Figure 4, Supplementary data S1- Table S6).

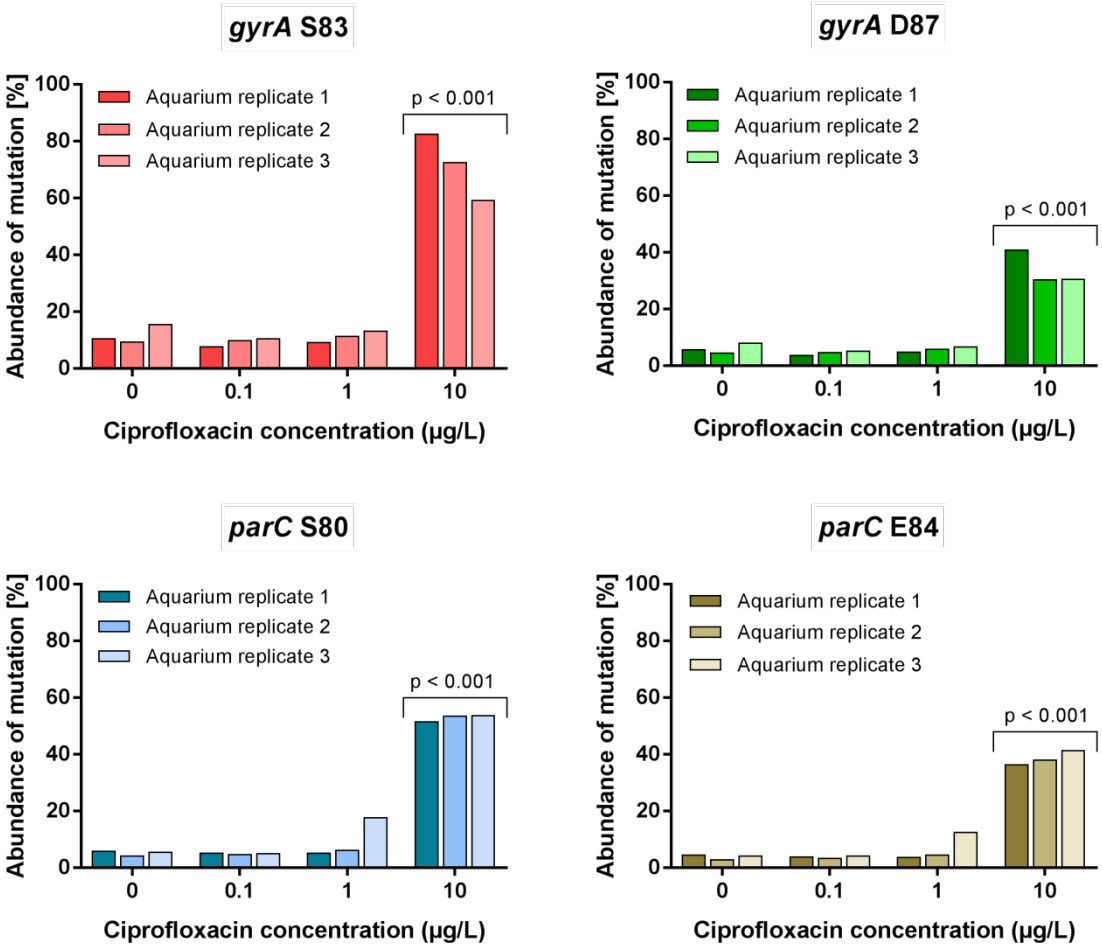

Figure 3 Relative abundance of mutations in *E.coli gyrA* codon S83 and D87 (upper panel) and *parC* S80 and E84 (lower panel) to total reads covering each DNA region in aquatic biofilms formed under different concentrations of ciprofloxacin. One-way ANOVA and Dunnett's post hoc test was performed using log-transformed read count ratios (mutated/codon coverage). Significant differences and corresponding p-values compared to control conditions (0 µg/L ciprofloxacin) are depicted.

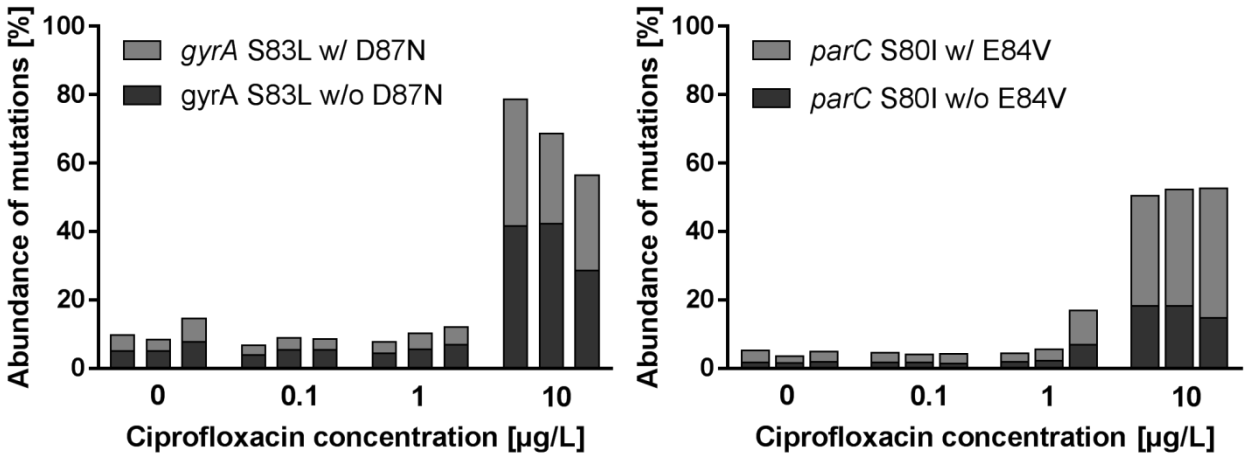

Figure 4 Relative abundance of mutations occurring in *E.coli gyrA* S83L with (w/) or without (w/o) D87N (right) and in *parC* S80I w/ or w/o E84V in aquatic biofilms formed under different concentrations of ciprofloxacin. Reads were normalized to the total read count covering both codons.

3.2.3.2. Allele specific PCR on E. coli isolates

To further investigate the genotypic profile of *E. coli* isolates in terms of co-occurrences of mutations in *gyrA* and *parC*, allele specific primers were designed to discriminate between substitutions in *gyrA*1 S83, *gyrA*2 D87 and *parC* S80. As only the highest ciprofloxacin exposure concentration had an effect on the abundance of mutations in *gyrA* and *parC*, solely isolates from selection tanks with no or 10 µg/L ciprofloxacin were further investigated. In total, 145 and 201 isolates that had been exposed to 0 or 10 µg/L, respectively, were randomly chosen from 0.25 mg/L ciprofloxacin containing plates, in order to be able to capture also genotypes that provide low-level resistance. To connect different genotypes to phenotypic ciprofloxacin resistance, MICs of selected isolates are depicted in Figure 5. Among all tested *E. coli* isolates more than 70 % had the triple combination *gyrA* S83L, D87N and *parC* S80I. This combination of substitutions confers a MIC > 32 µg/L. One other triple combination of *gyrA* S83L, D87Y and *parC* S80I resulted in a similar high MIC of > 32 µg/L. The MIC of isolates harboring *gyrA* S83L, D87E and *parC* S80I varied from 1.0 to 32 µg/L and were identified repeatedly. Approximately 8% of all tested isolates had no known resistance conferring mutation in neither of the investigated alleles, but MICs were between 0.75 and 1.0 µg/L, suggesting the involvement of other resistance mechanisms. Overall, no change in the prevalence of substitutions and combinations thereof could be observed between the *E.coli* harvested from control aquaria or from the highest ciprofloxacin exposure group (10 µg/L), noting that only *E. coli* growing on 0.25 mg/L ciprofloxacin containing agar plates were included in the comparison.

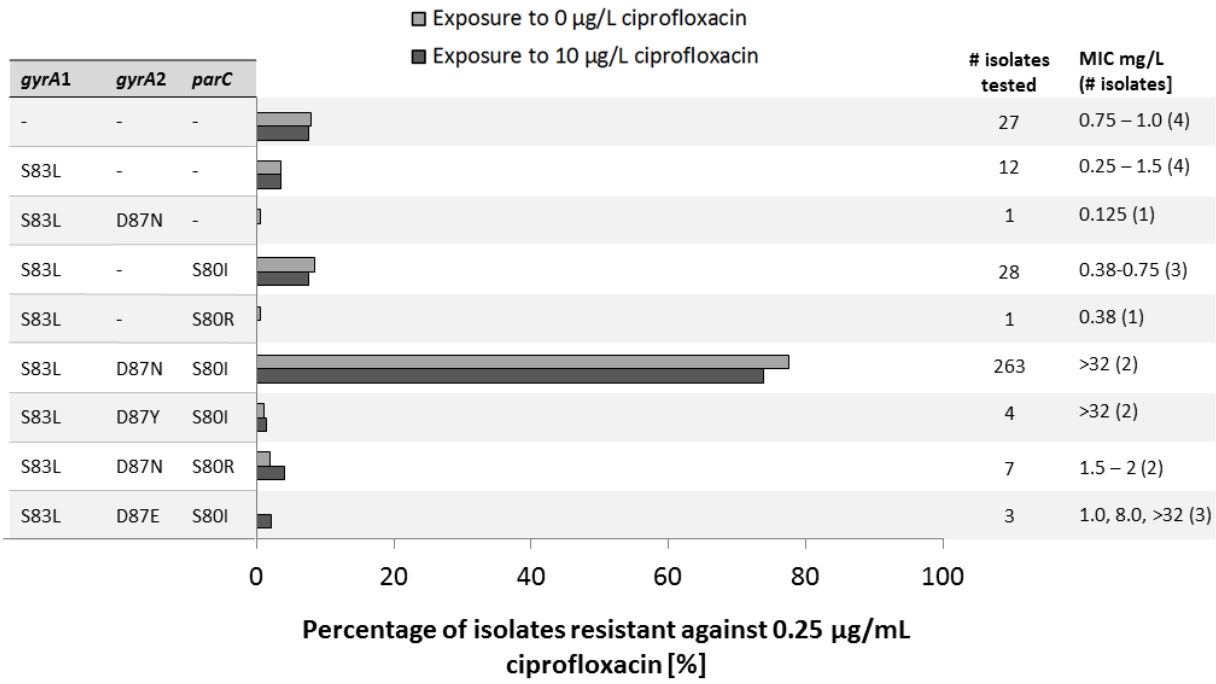

Figure 5 Genotypes of in total 145 and 201 *E. coli* colonies isolated from complex aquatic biofilm communities formed under 0 or 10 µg/L ciprofloxacin, respectively. *E. coli* grown on 0.25 mg/L ciprofloxacin containing agar plates were analyzed by allele specific PCR and screened for substitutions in *gyrA*1 S83, *gyrA*2 D87 and *parC* S80. The MIC was measured for a subset of randomly chosen isolates for each genotype.

3.2.4. Taxonomic analysis

Taxonomic composition was examined to identify a concentration that can exert selective pressure on the community as a consequence of ciprofloxacin exposure. 16S rRNA was extracted from metagenomic data and used for linear regression analysis. Genus diversity changed significantly across all exposure concentrations ($p = 0.00196$, linear regression Shannon diversity across all ciprofloxacin concentrations). To further investigate if both genus richness and evenness were affected by ciprofloxacin exposure, linear regression was applied to both parameters separately. Genus richness was unaffected ($p = 0.5713$, linear regression of genus richness across all ciprofloxacin concentrations), but evenness increased significantly ($p = 0.00147$, linear regression of evenness across all ciprofloxacin concentrations), indicating that certain genera are favored at the expense of others by ciprofloxacin exposure. This change in evenness was mainly driven by a reduction of *Pseudomonas*, the most prevalent genus in the biofilm (Table 3). At higher exposure levels, *Pseudomonas* decreased while *Bartonella*, *Ochrobactrum*, *Brucella*, *Pseudochrobactrum, Mesorhizobium, Leifsonia* increased in relative abundances. The Shannon diversity index revealed significant changes of genus diversity at 1 ($p = 0.00269$, Welch's two-sample t-test) and 10 µg/L ($p = 0.000267$, Welch's two-sample t-test) compared to the control conditions. Therefore, the LOEC for taxonomic changes was 1 µg/L. For individual taxa, the LOEC was 10 µg/L. It needs to be emphasized that our use of only three true biological replicates, high between sample variability and the explorative analysis setup used in this study could result in an underestimation of the effect concentration. At a more relaxed FDR cut-off of 0.1, where still 90% of the genes detected are expected to be true positives, changes in abundance already occur at 1 µg/L ciprofloxacin in some genera, ARGs and MGEs. P-values of the pairwise comparison without the Benjamini-Hochberg correction are given in the supplements (Supplementary data 1 – Table S7-S11).

Table 3 Bacterial classes and genera with significantly altered abundances by ciprofloxacin concentration (adjusted $p < 0.05$ after correcting for multiple testing using Benjamini-Hochberg false discovery rate) in complex aquatic communities exposed to different ciprofloxacin concentrations. Corresponding fold changes are provided in brackets.

Class						**Genus**						
	Adjusted p-value of regression analysis	Adjusted p-value pairwise comparison 0.1 µg/L		Adjusted p-value pairwise comparison 1 µg/L		Adjusted p-value pairwise comparison 10 µg/L			total counts	Adjusted p-value of regression analysis	Adjusted p-value pairwise comparison 0.1 µg/L		Adjusted p-value pairwise comparison 1 µg/L		Adjusted p-value pairwise comparison 10 µg/L	
Gamma-proteobacteria	0.003	0.90	(-1.1)	0.06	(-1.4)	0.01	(-2.0)	*Pseudomonas*	628275	0.006	0.93	(-1.1)	0.08	(-2.0)	0.01	(-2.8)
Alpha-proteobacteria	0.021	0.90	(1.2)	0.05	(1.7)	0.13	(1.9)	*Bartonella*	10172	0.004	0.91	(1.3)	0.27	(2.0)	0.04	(3.8)
								Ochrobactrum	25576	0.004	0.93	(1.1)	0.43	(1.8)	0.04	(3.9)
								Mesorhizobium	24441	0.006	0.91	(1.4)	0.59	(1.6)	0.04	(3.6)
								Pseudo-chrobactrum	45075	0.012	0.91	(1.3)	0.82	(1.7)	0.04	(4.6)
								Brucella	3499	0.006	0.95	(-1.0)	0.27	(1.9)	0.08	(3.7)
Actinobacteria	0.022	0.90	(1.4)	0.35	(1.5)	0.24	(2.7)	*Leifsonia*	404	0.012	0.91	(1.3)	0.43	(2.6)	0.14	(3.4)

3.2.5. Genotypic profiling of ARGs, MGEs and efflux pumps in complex aquatic biofilms

Ten quinolone resistance genes/gene clusters were detected in the samples. Three of these, namely *qnrB, qnrD* and *qnrS* were significantly and positively correlated to increasing ciprofloxacin concentrations (Table 4). The *qnrD* gene was significantly increased at both 1 and 10 µg/L, while *qnrB* and *qnrS* were significantly increased only at the highest concentration. For *qnrVC* and *aac(6')-Ib* there was a trend to an increase (adjusted p-value of regression analysis 0.079 and 0.086, respectively) and close to significance at 10 µg/L (adjusted p-value of pairwise comparison 0.0935). For the remaining five detected *qnr* gene clusters there were no clear trends. Notably, these were primarily genes predicted from metagenomics data (Boulund *et al.*, 2017).

Analysis of mobile ARGs in general revealed the presence of total 145 ARGs in the samples. In addition to the quinolone resistance genes addressed above, eight other ARGs followed significant ciprofloxacin exposure-response patterns, out of which two (MIR and OXA-48) were negatively correlated to ciprofloxacin concentration (Table 5).

Many ARGs are present as mobile gene cassettes in integrons (Recchia & Hall, 1995). The only integron with significant correlation to increased ciprofloxacin concentration was the environmental integron *intI6* (Table 6). Members of the class 1 and 2 integrons were less abundant in replicate samples of highest ciprofloxacin exposure levels than compared to control conditions. The *intI1* gene had comparably high read counts within the metagenomics data set, but the dose-response relationship trend was not significant according to the linear regression model (6417 total counts, adjusted p-value of regression analysis 0.077). Another mobile genetic element, the IS*CR*1 that is linked to class 1 integrons (Toleman *et al.*, 2006), had a 232.7 fold increased abundance at the highest exposure level. However, this remarkable

599 change of IS*CR*1 abundance was also characterized by an exceptionally high variance of read
600 counts within replicates for the highest exposure concentration (counts at 0 µg/L: 1; 3; 5
601 versus counts at 10 µg/L: 6; 633; 1098). Similar observations were made for IS*CR*3 (162.8
602 fold increased abundance at 10 µg/L; counts at 0 µg/L: 1; 0; 0 versus counts at 10 µg/L: 0; 84;
603 51).

604 The great majority of efflux pumps with significantly altered abundances at different exposure
605 levels were chromosomal RND efflux pumps (Table 7). Chromosomally encoded multidrug
606 efflux pumps belonging to the Mex system, present in the genus *Pseudomonas*, had overall
607 the highest read counts. The abundance of these Mex encoded efflux pumps significantly
608 decreased at the highest ciprofloxacin exposure levels, which was paralleled by a similar shift
609 of the genus *Pseudomonas* (Table 3). Another link between chromosomally encoded efflux
610 pumps and taxonomy shifts that occurred in response to ciprofloxacin exposure was observed
611 for RND efflux pumps encoded by *bepC*, *bepE* and *bepD* (Table 7). These pumps are
612 involved in extruding antimicrobial compounds in *Brucella* (Posadas *et al.*, 2007, Martin *et*
613 *al.*, 2009). Both abundance of *bepC*, *bepE*, *bepD* and the genus *Brucella* significantly
614 increased between 3.5 and 4.5 fold at 10 µg/L ciprofloxacin (Table 3 and Table 7).

615 **Table 4** Quinolone resistance genes detected in the metagenomic data set. Corresponding fold changes are provided in brackets.

Gene	Total counts[a]	Adjusted p-value of regression analysis[b]	Adjusted p-value pairwise comparison 0.1 µg/L		Adjusted p-value pairwise comparison 1 µg/L		Adjusted p-value pairwise comparison 10 µg/L	
qnrS	433	0.0074	0.476	(-1.0)	0.171	(1.4)	0.036	(3.0)
qnrB	688	0.0079	0.446	(-1.1)	0.099	(1.4)	0.036	(2.1)
qnrD	140	0.0079	0.446	(1.2)	0.028	(8.3)	0.009	(7.9)
qnrVC	1417	0.0793	0.446	(2.1)	0.196	(2.0)	0.094	(3.6)
aac(6')-Ib	645	0.0860	0.446	(1.7)	0.196	(2.0)	0.094	(106.1)
qnr_cluster_21	558	0.1444	0.446	(-1.3)	0.370	(-1.1)	0.137	(-1.6)
qnr_cluster_24	1157	0.1829	0.446	(-1.1)	0.082	(2.4)	0.333	(1.2)
qnr_cluster_8	662	0.1829	0.446	(1.2)	0.082	(1.6)	0.210	(1.2)
qnr_cluster_25	1098	0.2297	0.446	(-1.1)	0.068	(2.2)	0.480	(1.0)
qnr_cluster_12	936	0.3191	0.446	(-1.2)	0.196	(-1.2)	0.138	(1.1)

[a] Sum of counts over all samples and replicates. [b] Identical adjusted p-values are due to the Benjamini-Hochberg algorithm. It ensures that a p-values´ adjusted p-value will not be greater than a greater p-values´ adjusted p-value. Therefore, if a smaller adjusted p-value for a greater p-value is encountered, the adjusted p-values of all smaller p-values are set to that value.

616

617

Table 5 ARGs with significant changes in relative abundance with ciprofloxacin concentration (adjusted $p < 0.05$ after correcting for multiple testing using Benjamini-Hochberg false discovery rate) in complex aquatic communities exposed to different ciprofloxacin concentrations. Corresponding fold changes are provided in brackets.

Gene	Resistance to	Total counts[a]	Adjusted p-value of regression analysis[b]	Adjusted p-value pairwise comparison 0.1 µg/L		Adjusted p-value pairwise comparison 1 µg/L		Adjusted p-value pairwise comparison 10 µg/L	
MIR	beta-lactam	1376	0.0347	0.970	(-1.2)	0.118	(-2.0)	0.011	(-10.5)
qnrD	fluoroquinolone	140	0.0347	0.970	(1.2)	0.118	(8.3)	0.030	(7.9)
tet(39)	tetracycline	355	0.0347	0.970	(1.2)	0.695	(1.6)	0.055	(4.4)
qnrB	fluoroquinolone	688	0.0347	0.970	(-1.1)	0.322	(1.4)	0.127	(2.1)
qnrS	fluoroquinolone	433	0.0347	0.970	(-1.0)	0.454	(1.4)	0.139	(3.1)
catB2/catB3/catB10	chloramphenicol	497	0.0347	0.970	(1.0)	0.454	(1.5)	0.152	(2.8)
floR	chloramphenicol	3369	0.0347	0.970	(-1.1)	0.118	(2.2)	0.188	(2.6)
OXA-48	beta-lactam	823	0.0446	0.970	(1.2)	0.322	(-2.0)	0.064	(-16.3)
aph(3')-VIa	aminoglycoside	242	0.0446	0.970	(1.3)	0.460	(1.9)	0.148	(3.7)
cmlA	chloramphenicol	706	0.0446	0.970	(-1.0)	0.846	(1.2)	0.152	(9.7)
tet(E)	tetracycline	515	0.0446	0.970	(1.1)	0.322	(1.6)	0.188	(1.6)

[a] Sum of counts over all samples and replicates. [b] Identical adjusted p-values are due to the Benjamini-Hochberg algorithm. It ensures that a p-values´ adjusted p-value will not be greater than a greater p-values´ adjusted p-value. Therefore, if a smaller adjusted p-value for a greater p-value is encountered, the adjusted p-values of all smaller p-values are set to that value.

Table 6 Mobile genetic elements with significant changes in relative abundance with ciprofloxacin concentration (adjusted $p < 0.05$ after correcting for multiple testing using Benjamini-Hochberg false discovery rate) in complex aquatic communities exposed to different ciprofloxacin concentrations. Corresponding fold changes are provided in brackets.

Gene	Total counts[a]	Adjusted p-value of regression analysis[b]	Adjusted p-value pairwise comparison 0.1 µg/L		Adjusted p-value pairwise comparison 1 µg/L		Adjusted p-value pairwise comparison 10 µg/L	
ISCR8	10638	0.020	0.464	(1.3)	0.251	(1.2)	0.009	(1.9)
intI6	185	0.020	0.464	(1.7)	0.088	(2.8)	0.115	(5.2)
intI2	310	0.028	0.798	(1.1)	0.351	(-1.4)	0.023	(-12.5)
ISCR3	149	0.028	0.464	(7.2)	0.088	(8.3)	0.081	(162.8)
ISCR14	557	0.028	0.464	(1.9)	0.303	(2.7)	0.115	(7.0)
ISCR1	1815	0.040	0.464	(2.5)	0.121	(6.6)	0.081	(232.7)

[a] Sum of counts over all samples and replicates. [b] Identical adjusted p-values are due to the Benjamini-Hochberg algorithm. It ensures that a p-values´ adjusted p-value will not be greater than a greater p-values´ adjusted p-value. Therefore, if a smaller adjusted p-value for a greater p-value is encountered, the adjusted p-values of all smaller p-values are set to that value.

Table 7 Efflux pumps with significant changes in relative abundance with ciprofloxacin concentration (adjusted $p < 0.05$ after correcting for multiple testing using Benjamini-Hochberg false discovery rate) in complex aquatic communities exposed to different ciprofloxacin concentrations. Corresponding fold changes are provided in brackets.

Gene	Total counts[a]	Adjusted p-value of regression analysis[b]	Adjusted p-value pairwise comparison 0.1 µg/L		Adjusted p-value pairwise comparison 1 µg/L		Adjusted p-value pairwise comparison 10 µg/L	
bepE	33381	0.006	0.965	(1.1)	0.212	(1.6)	0.017	(3.5)
bepC	3742	0.006	0.986	(-1.0)	0.233	(1.5)	0.018	(3.5)
bepD	5543	0.006	0.965	(-1.2)	0.212	(1.9)	0.024	(4.5)
mexF	166841	0.008	0.965	(-1.1)	0.195	(-1.8)	0.017	(-2.8)
ttgB	128323	0.012	0.965	(-1.4)	0.212	(-2.0)	0.051	(-2.7)
tet(39)	355	0.017	0.965	(1.2)	0.637	(1.6)	0.017	(4.4)
silA	49393	0.019	0.965	(-1.1)	0.269	(-1.3)	0.009	(-3.5)
adeG	22309	0.019	0.965	(1.2)	0.212	(1.4)	0.056	(2.1)
mexE	41653	0.019	0.965	(-1.5)	0.233	(-2.1)	0.066	(-3.2)
ttgA	37326	0.019	0.965	(-1.5)	0.233	(-2.4)	0.073	(-3.1)
ttgC	47340	0.019	0.965	(-1.5)	0.233	(-2.3)	0.073	(-3.1)
arsB	20183	0.025	0.965	(-1.1)	0.496	(-1.2)	0.013	(-2.7)
smfY	734	0.025	0.965	(-1.1)	0.553	(1.3)	0.051	(2.5)
rcnA/yohM	1556	0.026	0.965	(-1.2)	0.233	(-1.3)	0.027	(-2.0)
floR	3133	0.026	0.965	(-1.2)	0.116	(2.2)	0.103	(2.6)
mexK	97585	0.030	0.965	(1.1)	0.269	(-1.6)	0.066	(-2.3)
cmlA	702	0.030	0.965	(-1.1)	0.786	(1.2)	0.068	(9.7)
tet(E)	515	0.030	0.965	(1.1)	0.237	(1.6)	0.095	(1.6)
mexW	96883	0.037	0.965	(1.1)	0.237	(-1.8)	0.066	(-2.5)

acrA	5490	0.039	0.965	(-1.1)	0.685	(-1.2)	0.049	(-3.1)
gesA	157	0.039	0.965	(-1.4)	0.393	(-1.4)	0.073	(-1.8)
gesB	28374	0.040	0.965	(1.2)	0.419	(1.3)	0.073	(2.2)
smrA	46519	0.041	0.965	(1.0)	0.116	(1.4)	0.097	(1.2)
abeM	747	0.045	0.965	(1.7)	0.237	(6.0)	0.003	(6.8)

[a] Sum of counts over all samples and replicates. [b] Identical adjusted p-values are due to the Benjamini-Hochberg algorithm. It ensures that a p-values´ adjusted p-value will not be greater than a greater p-values´ adjusted p-value. Therefore, if a smaller adjusted p-value for a greater p-value is encountered, the adjusted p-values of all smaller p-values are set to that value.

4. Discussion

To understand risks associated with antibiotics in the environment it is central to clarify *how* environmental selection pressures could lead to increased problems with infections caused by resistant bacteria. Conceptually, such selection pressures likely play a very minor role for the environmental *transmission of already resistant pathogens* of most species. Other transmission barriers are arguably much more important determinants for the ability of the pathogens to move from a habitable patch within a human host, via an environmental, hostile dispersal matrix where growth opportunities are limited, to reach another human host in sufficient numbers to cause an infection (Bengtsson-Palme *et al.*, 2017). Exceptions could apply to pathogenic species that primarily grow and reside in the external environment (such as *Legionella*), where small selective advantages over many generations caused by antibiotics or co-selective agents plausibly could influence the proportion of infections with antibiotic resistant strains. The risk that environmental selection pressures favor the *mobilization, enrichment and transfer of novel resistance determinants* to human pathogens or intermediate hosts, is on the other hand, a scenario that has much broader applicability, and with potential consequences not only for the number of infections with resistant bacteria, but also for our future ability to treat infections globally in the long term (Bengtsson-Palme & Larsson, 2015, Boulund *et al.*, 2017). Direct measures of the selection of such novel resistance factors are difficult to obtain, simply because those genes are not known. The critical question is then how well other endpoints are likely to reflect such a selection pressure? Below, we discuss the informative value of the range of endpoints measured in this study, using ciprofloxacin as an example.

A complex community is characterized by the parallel existence of different genotypes. Antibiotic exposure can therefore result in selection of several different resistance mechanisms, regardless if acquired or intrinsic to all members of a species. One of our aims

was to carefully examine the resistance mechanisms that are predominantly selected for in complex communities upon ciprofloxacin exposure. Chromosomal resistance mutations were selected for, but only at 10 µg/L. The prevalence of mutations in the QRDR of *E. coli* revealed that the vast majority of the resistant isolates harbored triple mutations in *gyrA* S83L/D87N and *parC* S80I. As shown earlier, combination of mutations can lead to reduced fitness cost. However, also single mutations in *gyrA* and *parC* or combinations thereof have comparably small fitness costs (Marcusson *et al.*, 2009). The use of a ciprofloxacin containing screening agar for *E. coli* isolates to identify mutations on both alleles, *gyrA* and *parC*, may have resulted in an underestimation of single mutants with comparably low MICs. But enrichment of strains with any chromosomal resistance mutations in a controlled exposure setup like the one used here is clearly indicative of a selection pressure, relevant also for the selection of unknown resistance factors. However, such an extrapolation assumes that the unknown mobile resistance factor of concern would bear similarly low cost and provide similar resistance at the tested concentration as the chromosomal mutations studied. The human health risks associated with environmental selection for chromosomal mutations *per se* is probably small, as such mutations would not be readily transferrable, and much stronger selection pressures favoring such mutations in pathogens would occur relatively frequently in the gut of humans and domestic animals anyway.

Based on that both unknown and known mobile resistance genes are likely selected for together with other genes on mobile elements (such as plasmids), which often may incur a significant fitness cost on growth, one might argue that selection of known mobile resistance genes would be somewhat more informative than chromosomal mutations. When *E. coli* isolates were tested for combinations of mutations on both alleles, *gyrA* and *parC*, we identified 8% that had no mutation (Figure 5) while corresponding MICs (0.75 – 1.0 mg/L) of these isolates were higher than the EUCAST clinical breakpoint for *Enterobacteriaceae* (S ≤

683 0.25 mg/L, R > 0.5 mg/L) and the epidemiological cut-off value for *E. coli* (S ≤ 0.064 mg/L)
684 (EUCAST, 2016). The observed, acquired resistance is therefore caused by other mechanism,
685 such as mobile *qnr* genes or upregulated efflux pumps (Redgrave *et al.*, 2014). Although there
686 is a wide range of known *qnr* genes, three genes, *qnrB, qnrD* and *qnrS,* dominated in the fecal
687 flora of Swedish study subjects (Rutgersson *et al.*, 2014). Exactly these three *qnr* genes were
688 significantly enriched with an increasing exposure to ciprofloxacin in this study, likely as
689 direct consequence of the resistance they provide to their hosts. The *qnrD* gene was
690 significantly increased already at 1 µg/L. We interpret this as a strong indication that also
691 other, unknown, mobile resistance factors could be selected for at this concentration.

692 At the same time, we would like to raise a warning about over-interpreting apparently random
693 changes of ARG abundances in studies of communities, as many of these are likely to simply
694 be consequences of taxonomic shifts. For example, the decreased prevalence of the beta-
695 lactamase genes *MIR* and *OXA-48* that are increasingly reported in *Enterobacteriaceae*
696 (Jacoby, 2009, Poirel *et al.*, 2012) correlate with a decline of the *Enterobacteriaceae* family at
697 a ciprofloxacin exposure concentration of 10 µg/L (18,813 total counts at 0 µg/L vs 7,701
698 total counts at 10 µg/L ciprofloxacin, linear regression p = 0.654, pairwise comparison after
699 correction for multiple testing p = 0.027). Furthermore, *Acinetobacter baumanii* is a known
700 carrier of *tet(39)* and *aph(3')-VIa*. An increased prevalence of the genus *Acinetobacter* at
701 highest exposure levels might explain the more abundant occurrence of *tet(39)* and *aph(3')-*
702 *VIa* in these samples (3,258 total counts at 0 µg/L vs 13,992 total counts at 10 µg/L
703 ciprofloxacin, linear regression p = 0.1353, pairwise comparison after correction for multiple
704 testing p = 0.0125). Similar observations were made for efflux pumps. Alterations in the
705 prevalence of *mex*- or *bep*-encoded efflux systems were paralleled by shifts of *Pseudomonas*
706 and *Brucella*, which are known carriers of these pumps. Hence, changes in the ARG, MGE
707 and efflux pump profiles that are not directly associated with quinolone resistance often seem

to be secondary effects caused indirectly by taxonomic shifts. In uncontrolled setups, where other factors than the selective agent varies between sample groups, it is even more challenging to interpret changes in gene abundances, as taxonomic composition can be affected by numerous factors unrelated to antibiotics.

Changes in taxonomy in controlled experiments where only the antibiotic concentration is varied indicates that the antibiotic has limited the growth of at least some species in the community. The EUCAST database reports MIC distributions of down to 2 μg/L for ciprofloxacin. Such low MICs for ciprofloxacin are reported for selected strains of *Enterobacter cloacae*, *E. coli*, *Haemophilus influenzae*, *Helicobacter pylori*, *Neisseria gonorrhoeae* and *Neisseria meningtidis*. Noting that the MIC represents a concentration that *completely* inhibits growth of the studied strain, the sensitivity spectrum of these species and other bacteria present in the environment might be even lower. The observed LOEC of 1 μg/L ciprofloxacin that causes taxonomy shifts is just below the lowest MICs reported in EUCAST (EUCAST, 2016). This link between the antibiotic concentration that selects for resistance (LOEC) within a complex community and the one inhibiting bacterial growth (MIC) can be used to estimate upper boundaries for selective concentrations for a wide range of antibiotics as demonstrated by Bengtsson-Palme and Larsson (2016). This theoretical approach is based on the assumption that selective concentrations need to be lower than those completely inhibiting growth, at least in some communities, although how much lower depends on the cost of carrying the resistance factor (Bengtsson-Palme & Larsson, 2016). The estimated sample size-adjusted lowest MIC of 1 μg/L for ciprofloxacin corresponds to the experimentally determined LOEC in this study. In a similar effort, Tello et al. used species sensitivity distributions from the EUCAST database, predicting a NOEC for selection as low as 0.1 μg/L (Tello *et al.*, 2012), in line with our experimental derivation.

One may also reflect on if it matters which species are subjected to a selection pressure. We argue that it can very well matter. Even though enrichment of a potential donor of a resistance factor may increase the likelihood for a transfer to a pathogen, the most critical aspect is that there is a selection pressure on the receiver, favoring those initially very rare cells that have acquired resistance. From this reasoning, it would be more relevant if there is a selection pressure on a human pathogen (or a commensal, acting as an intermediate host) than on species that are not part of the human microbiota. Since the EUCAST database primarily cover clinically relevant bacteria, selective concentration from that data (Bengtsson-Palme and Larsson, 2016) seems relevant from the described risk scenario. In the present study, the resolution of taxa could not reach down to species with certainty, even less so differentiating between virulent and non-virulent strains. *Pseudomonas* was identified as the most clearly affected genus. Infections with *P. aeruginosa* have become a very common cause for nosocomial infections, particularly in immune-compromised patients. Hence, a direct environmental antibiotic selection pressure on *Pseudomonas* species could very well be relevant from a clinical point of view. However, *P. aeruginosa* is, according to EUCAST, not among the species that are particularly sensitive to ciprofloxacin. It is therefore quite possible that the observed decline in the relative *Pseudomonas* abundance with increasing ciprofloxacin concentrations is an ecological downstream effect, rather than a consequence of a direct selection pressure from the antibiotic on this genus. If so, the exposure would also not favor acquired ciprofloxacin resistance in *Pseudomonas* as it is likely other features than its direct sensitivity to the antibiotic that governed its decline here.

Within species selection of strains with acquired resistance is the most concerning and relevant form of selection (Pal *et al.*, 2017). Still, most previous environmental studies on communities, including our own, have not assessed within species selection directly. Increases of resistant strains over non-resistant could encompass a range of resistance

757 mechanisms, known and novel. Here, we showed that 10, but not 1 µg/L increased the
758 proportion of resistant *E. coli* in the flow-through systems, and 5, but not 1 µg/L in the test-
759 tube system. A possible reason to why this endpoint apparently was slightly less sensitive
760 than the analyses of *qnrD* is that we only investigated one species, and *E. coli* is quite
761 plausibly not the most sensitive member of the community, nor the main carrier of *qnrD*. On
762 the other hand, we investigated several quinolone resistance genes to identify the most
763 sensitive indicator. As indicated above, changes in mobile genes can also be a reflection of
764 indirect between species selection. Therefore, we cannot with certainty conclude that the
765 increase in *qnrD* (also) reflects within species selection. Still we think it is very likely based
766 on its superior sensitivity to ciprofloxacin compared to other non-quinolone resistance genes.
767 In addition, all three of the detected, well-studied *qnr* gene clusters that are commonly found
768 in Swedish fecal microbiota as well as human pathogens, show a significant, positive
769 correlation with increasing antibiotic concentrations, similar as was the case for *tet*-genes in
770 response to tetracycline (Lundström *et al.*, 2016). These observations are all in line with a
771 direct advantage from carrying acquired *qnr* genes, rather than indirect selection of species
772 that by chance happen to carry them.

773 The more elaborate biofilm flow-through system gave similar results as the simple planktonic
774 test-tube system (NOEC = 1 µg/L for the selection of resistant *E. coli*). This could make the
775 test-tube system with communities attractive for screening, but we should acknowledge that
776 we did not evaluate it for the endpoints that turned out to be the most sensitive ones in the
777 flow-through system, i.e. *qnrD* and taxonomic diversity. On the downside, the strong and
778 rapid reduction in complexity in the test tubes (Supplementary data 1 - Figure S1) makes it
779 considerably less representative of environmental communities. The overall NOEC from the
780 biofilm flow through setup was 0.1 µg/L. This is identical to the MSC estimated by Gullberg
781 et al. (2011). They measured selection down to 0.23 µg/L which is slightly lower than what

782 we found for the endpoints *taxonomic diversity* and *qnrD* and clearly lower than the selection
783 of resistant *E. coli* within the communities. A plausible explanation is that the selective ability
784 in fact may differ between the two-strain competition system and complex communities, by
785 one or several of the mechanisms described in the introduction. For example, Suci et al.
786 demonstrated that transport of ciprofloxacin in biofilms was strongly impeded by *P.*
787 *aeruginosa* (Suci *et al.*, 1994). In this study, *Pseudomonas* was the most abundant genus in
788 the biofilms, which may have indirectly protected other species. Some of the differences may
789 also lie in the number of generations studied, as minor growth advantages needs many
790 generations to become detectable. The dilution rate theoretically sets the growth rate in a
791 continuous culture under steady state conditions (Monod, 1950). The dilution rate of the
792 aquaria test system used in this study is comparably low with 0.0616 h^{-1}. Gullberg et al. based
793 their competition experiments on ca 40 generations with ca 10 generations per 24 h using
794 nutrient rich medium (Gullberg *et al.*, 2011). The diluted R2A medium used in this study
795 contains only reduced levels of nutrients, non-optimal for fast growing species (van der Linde
796 *et al.*, 1999). An attempt to estimate the number of generations of *E. coli* generations in our
797 flow through set up resulted in approximately 30 (supplementary data 1) but the estimate is
798 uncertain and should be viewed as a theoretical maximum, particularly as the bacteria that
799 colonize glass slides and form biofilms may grow slower than the planktonic fraction. Hence,
800 had we continued the experiment over considerably longer time, we cannot exclude that
801 effects could have been detected at concentrations down to those found by Gullberg et al.
802 (2011).

803 Based on the above, our derived LOEC for ciprofloxacin selection here was 1 µg/L and a
804 NOEC of 0.1 µg/L. European surface waters rarely exceed this NOEC, indicating minor risks
805 for selection (Thomas *et al.*, 2007, Zuccato *et al.*, 2010), also supported by concentrations
806 commonly found in treated effluents of European WWTPs (Lindberg *et al.*, 2005, Singer *et*

al., 2014, Bengtsson-Palme *et al.*, 2016). Influent concentrations are typically between 0.1 and 1 µg/L (Lindberg *et al.*, 2005, Bengtsson-Palme *et al.*, 2016, Ostman *et al.*, 2017), suggesting some potential for selection within treatment plants. Levels in untreated hospital effluents can be even higher, up to 17 µg/L as reported in France (Ory *et al.*, 2016). To this should be added the expected additive effects of co-exposure to several fluoroquinolones (Backhaus *et al.*, 2000, Menz *et al.*, 2017). However, industrially-polluted environments stand out with up to mg/L concentrations (Larsson *et al.*, 2007, Fick *et al.*, 2009, Gothwal & Shashidhar, 2017).

To our best knowledge, there are no studies demonstrating selection of quinolone resistant bacteria in surface waters contaminated by excreted antibiotics. Correlative studies exist, but they bear limited value as both the resistant bacteria and the antibiotics often originate from the same source (urine/feces from humans and domestic animals). Some studies on resistant/sensitive isolates suggest selection within treatment plants (Ferreira da Silva *et al.*, 2007, Lefkowitz & Duran, 2009), whereas other studies do not (Reinthaler *et al.*, 2003, Osinska *et al.*, 2017). Most of these studies suffer from a low number of studied isolates, a strategy based on a few grab samples, and/or a lack of measurements of antibiotics, making interpretation and conclusions ambiguous. Other studies, following the abundance of ARGs through the sewage treatment process, observed no enrichment of *qnr* genes (Bengtsson-Palme *et al.*, 2016, Auguet *et al.*, 2017). Similarly, Rutgersson et al. found no enrichment of *qnr* genes in Indian well water polluted with fluoroquinolones up to 0.915 µg/L (sum of total quinolones) (Rutgersson *et al.*, 2014). In environments polluted by drug manufacturing, fluoroquinolone levels may be so high that basically only resistant bacteria can survive. Accordingly, the communities are characterized by high abundances of *qnr* genes (Kristiansson *et al.*, 2011, Rutgersson *et al.*, 2014) and quinolone resistant bacteria (Flach *et al.*, 2015), which are often multiresistant (Marathe *et al.*, 2013).

Based on current knowledge, we therefore believe 0.1 μg/L is a reasonable exposure limit for ciprofloxacin to prevent environmental selection or at least keep it very limited. From a strict precautionary perspective one could argue for an even lower discharge or surface water limit (Nijsingh *et al.*, 2018), but it would then come with significantly increased infrastructure needs. At the same time, concentrations and potential resistance selection inside sewage treatment plants will be difficult to reduce by practical means other than reduction in use and/or disinfection of effluents to prevent release of resistant bacteria. More research is clearly needed to investigate if selection occurs within sewage treatment plants. For certain hospital wastes, selection seems plausible given exposure levels, and in some industrially polluted environments, there is with certainty a strong selection. This calls for a mixture of direct actions to limit pollution as well as more research (Review on Antimicrobial Resistance, 2016, UNEP, 2016, JPIAMR, 2017, Bengtsson-Palme *et al.*, 2018).

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Supplementary Data

Supplementary data 1 – Figure S1, Table S1- S13

Supplementary data 2 – qnr clusters

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