NOVEL INSIGHTS INTO SELECTION FOR ANTIBIOTIC RESISTANCE IN COMPLEX MICROBIAL COMMUNITIES

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Abstract word count: 178

Text word count (excluding table footnotes and figure legends): 5,102

ABSTRACT

Recent research has demonstrated selection for antibiotic resistance occurs at very low antibiotic concentrations in single species experiments, but the relevance of these findings when species are embedded in complex microbial communities is unclear. We show the strength of selection for naturally occurring resistance alleles in a complex community remains constant from low sub-inhibitory to above clinically relevant concentrations. Selection increases with antibiotic concentration before reaching a plateau where selection remains constant over a two order magnitude concentration range. This is likely to be due to cross-protection of the susceptible bacteria in the community following rapid extracellular antibiotic degradation by the resistant population, shown experimentally through a combination of chemical quantification and bacterial growth experiments. Metagenome and 16S rRNA analyses on sewage-derived bacterial communities evolved under cefotaxime exposure show preferential enrichment for $bla_{\text{CTX-M}}$ genes over all other beta-lactamase genes, as well as positive selection and co-selection for antibiotic resistant, opportunistic pathogens. These findings have far reaching implications for our understanding of the evolution of antibiotic resistance, by challenging the long-standing assumption that selection occurs in a dose-dependent manner.

IMPORTANCE

Antibiotic resistance is one of the greatest, global issues facing modern society. Still, comparatively little is known about selection for resistance at very low antibiotic concentrations. We show that the strength of selection for clinically important resistance genes within a complex bacterial community can remain constant across a large antibiotic

concentration (wide selective space). Therefore, largely understudied ecological compartments could be just as important as clinical environments for selection of antibiotic resistance.

INTRODUCTION

Antibiotic resistance poses a major threat to society, the sustainability of modern healthcare systems, food security and the global economy [1, 2]. Until recently, most research on evolution of resistance focused on selection at clinically relevant antibiotic concentrations, as the 'traditional' selective window hypothesis was universally accepted. This hypothesis states that selection for antibiotic resistance will only occur above the minimum inhibitory concentration (MIC) of susceptible bacteria and below the MIC of resistant bacteria [3]. In fact, numerous experimental studies have observed selection for resistance at sub-MIC antibiotic concentrations, at the point where the selective pressure (antibiotic) is sufficient to offset the cost of resistance [3-7]. In recent isogenic studies, a single host species with chromosomal or plasmid-borne resistance mechanisms were competed with their susceptible counterparts at varying concentrations of antibiotic to determine the minimal selective concentration (MSC) [3, 5]. The MSC is the lowest concentration of antibiotic at which resistance is positively selected, which can be significantly lower than the minimum inhibitory concentration (MIC) [3, 5]. MSCs have also been estimated using publically available, clinical breakpoint data [8] but experimental data is required to assess the validity of these predictions, especially in a community context. These findings show that the selective compartment (meaning the antibiotic gradient and spatial range along which resistant bacteria/genes could be enriched) is much larger than previously thought [9]. This in turn

suggests selection may be occurring in previously unconsidered selective compartments which harbour relatively low antibiotic concentrations, such as the gut microbiome, waste water and even surface waters contaminated with antibiotic residues.

Though these findings are significant, the use of single species means their relevance with regards to selection in complex microbial communities remains unclear. Many studies have quantified numbers and/or prevalence of resistance genes in waste water influent and effluent (including, but not limited to [10, 11]), with a recent study utilising epicPCR to identify the host background of highly abundant resistance genes [12]. However, positive selection for resistance within complex bacterial communities is a current knowledge gap [13], with experimental MSC data in complex communities severely lacking. One recent study [14] reported a biological effect at low concentrations of tetracycline in a microbial community, by quantifying tetracycline resistance gene prevalence (*tetA* and *tetG* genes normalised to 16s rRNA copy number). However, as starting gene frequencies were not measured, it is unclear if the observed effect was driven by positive selection; or reduced negative selection. In other words, without comparing the final resistance gene prevalence to the initial resistance gene prevalence, it is unknown if resistance genes actually increased over time under tetracycline exposure (i.e. were positively selected); or if resistance genes were simply lost at a slower rate compared to the no antibiotic control (i.e. were negatively selected, or showed increased persistence). Here, we aimed to quantify positive selection in a complex bacterial community by conducting evolution experiments using a waste water bacterial community inoculum to determine the MSC of cefotaxime. Co-selection for other resistance genes and effects on community structure were also determined through metagenome analyses.

Cefotaxime is a World Health Organisation (WHO) recognised 'critically important' antibiotic [15], 'essential' for human medicine [16] that was most recently identified as a key antimicrobial stewardship target through inclusion in the WHO 'watch list' of essential medicines [17]. In this study, prevalence of the $bla_{\text{CTX-M}}$ gene group was determined with qPCR and selection coefficients were calculated to estimate the MSC of cefotaxime. CTX-Ms are extended spectrum beta-lactamases (ESBLs) which cleave the beta-lactam ring, effectively inactivating and degrading beta-lactam antibiotics [18]. Previous work has demonstrated beta-lactamases can inactivate extracellular beta-lactams, to the benefit of nearby susceptible bacteria [19-21]. This protective effect on susceptible bacteria has since been shown for an intracellularly expressed resistance mechanism, also degradative in nature [22].

Results show, for the first time, that selection for $bla_{\text{CTX-M}}$ genes occurs at very low, sub-inhibitory concentrations. We also demonstrate that selection occurs with equal potency at very low antibiotic concentrations and at concentrations greatly exceeding those used in the clinic. Therefore, antibiotic resistance is not always selected for in a dose-dependent manner. These findings illustrate the importance of studying selection for resistance within complex bacterial communities over a wide selective range, representative of different selective compartments [9].

RESULTS

Cefotaxime exposure affects community structure. Complex community (raw, untreated waste water) microcosms were spiked with a range of concentrations of cefotaxime. The exposure concentration range was selected from the EUCAST [23] defined clinical breakpoint

concentration (the concentration at which *Enterobacteriaceae* are considered 'clinically' resistant) down to 0, in a two-fold dilution series including concentrations similar to those previously measured in different environments (such as hospital effluent, waste water treatment plant effluent and surface waters (low µg/L up to 150 µg/L [24-26])). Bacterial communities were transferred daily into fresh medium and fresh antibiotic for 8 days. Chemical quantification was performed at the beginning of day 0 and after 24 hours, to determine an accurate MSC (based on measured rather than nominal antibiotic concentration) and to assess the chemical stability of cefotaxime (a third generation cephalosporin of the beta-lactam class of antibiotics) in the system.

At the end of the experiment, three replicates from a low, medium and high antibiotic concentration were selected to undergo metagenome analyses, alongside the unexposed control to identify the key resistance genes under selection. Metagenomic DNA was sequenced with Illumina MiSeq2.

16S rRNA data was extracted from trimmed, quality controlled paired reads with MetaPhlan2 [27]. Overall, the community comprised of predominantly Gram negative bacteria, though Gram positive bacteria were also detected (Figure S1). Between-sample variation was expected due to unavoidable heterogeneity within the complex community inoculum. Even so, there were clear differences between the control untreated bacterial community and the communities exposed to cefotaxime amongst the 25 most abundance species (Figure 1, all detected species can be seen in Figure S1). In particular, several species were eliminated by cefotaxime treatment (or reduced below the limit of detection). These included the opportunistic Gram negative pathogens *Providencia alcalifaciens, Aeromonas veronii, Morganella morganii* and *Klebsiella pneumoniae*; as well as the opportunistic Gram positive

pathogen *Streptococcus infantarius*. All of these were significantly associated with the no antibiotic control as determined by Linear Discriminant Analyses (LDA) effect size (LEfSe) analyses (Figure S2). Conversely, several Gram negative and Gram positive opportunistic pathogens showed greater abundance in treated communities compared to the untreated control; namely *Pseudomonas aeruginosa, Acinetobacter baumanii*, *Bacteroides fragilis* and *Enterococcus faecalis*; however, only *P. aeruginosa* was significantly enriched in the 2 mg/L cefotaxime treatment (LEfSe, Figure S2). Cefotaxime treatment also resulted in slightly decreased numbers of *Escherichia coli*, though this was not significant and it was still the predominant species across all treatments. In general, there was much greater variability between treatment replicates compared to the untreated control (Figure S1).

***Bla*$_{\text{CTX-M}}$ genes are preferentially selected over all other beta-lactam resistance mechanisms.** Metagenomic data was further analysed with the ARGs-OAP pipeline [28], designed to thoroughly interrogate metagenomic data and identify resistance genes. Selection for beta-lactam resistance was prominent (as expected); however, co-selection for resistance to unrelated antibiotic classes was also observed: namely, co-selection for resistance to macrolides, aminoglycosides, trimethoprim, tetracyclines and sulphonamides which is likely to be due to carriage of multi-resistance plasmids (see Figure S3).

We delved deeper into the beta-lactam resistance genes to determine which genes, if any, were preferentially selected. We observed substantial enrichment for the beta-lactamase and extended-spectrum beta-lactamase (ESBL) genes *bla*$_{\text{TEM}}$, *bla*$_{\text{OXA}}$ and *bla*$_{\text{CTX-M}}$ (Figure 2). Average increases in relative abundance from the lowest to highest concentration were 8-fold, 8-fold and 70-fold, respectively. *Bla*$_{\text{CTX-M}}$ was preferentially selected over all other beta-lactamase encoding genes at each cefotaxime concentration.

The MSC of cefotaxime is very low, but selection plateaus across a large concentration range. Given the strong positive selection for *bla*$_{\text{CTX-M,}}$ we focused on accurate quantification of this group of genes across the entire experimental antibiotic gradient using qPCR. This follows previous work which showed qPCR is the most sensitive method for MSC determination [14]. *Bla*$_{\text{CTX-M}}$ gene copy number was normalised to 16S rRNA copy number, to determine a molecular 'prevalence' of *bla*$_{\text{CTX-M}}$; this prevalence was determined for each cefotaxime concentration both at the beginning and end of the experiment. A Kruskal-Wallis test confirmed *bla*$_{\text{CTX-M}}$ prevalence, 16S rRNA copy number and *bla*$_{\text{CTX-M}}$ copy number (all n=5 each) did not differ significantly between treatments at day 0.

Selection coefficients based on change in *bla*$_{\text{CTX-M}}$ prevalence over time were calculated and plotted against cefotaxime concentration as in previous single species assays [3, 5] (Figure 3). A positive selection coefficient value indicates positive selection is occurring, and the x-axis intercept estimates the MSC; here, 0.4µg/L (Figure S4).

Bla$_{\text{CTX-M}}$ prevalence increased over time (Figure S5) and with antibiotic concentration (linear term: $F_{1,}$ 42= 26.3, $P < 0.001$), but appeared to plateau at 500µg/L (quadratic term: F_1, 42 = 13.2, $P < 0.001$) so an additional experiment was performed to determine if this plateau continued at higher concentrations (Figure 3). As hypothesised, *bla*$_{\text{CTX-M}}$ prevalence increased when exposed to cefotaxime (linear term: F_1, 36 = 9.6, P <0.01) but remained relatively constant (quadratic term: F_1, 36 = 9.4, P <0.01) up until the two highest concentrations used in this study (Figure 3). These concentrations are over 30x and 50x times the defined clinical breakpoint cefotaxime concentration of 2 mg/L for *Enterobacteriaceae*. The rise in *bla*$_{\text{CTX-M}}$ prevalence at 64 mg/L was due to an increase in *bla*$_{\text{CTX-M}}$ gene copy number, and the decrease

at 128 mg/L was due to a significant decrease in *bla*$_{\text{CTX-M}}$ and slight reduction in 16S rRNA copy number (Figures S6 and S7).

The bacterial community readily degrades cefotaxime. We hypothesised that this plateau in selection was due to both the mechanism and sociality of the *bla*$_{\text{CTX-M}}$ genes: as beta-lactamase enzymes can be found both intracellularly and extracellularly [21], the plateau in *bla*$_{\text{CTX-M}}$ prevalence may be due to negative frequency-dependent selection [19]. In other words, the more prevalent *bla*$_{\text{CTX-M}}$ becomes, the lower its fitness as cefotaxime degradation is accelerated to the benefit of the entire community, including non-*bla*$_{\text{CTX-M}}$ bearing competitors. To investigate if cefotaxime was degraded by the community, chemical quantification of cefotaxime in the presence of the community was performed. Incubating the microcosms for 24 hours resulted in complete degradation of cefotaxime, at all but the highest concentration (Table S1). All measured concentrations were lower than expected, and the lowest concentration (15.625 µg/L) was below the limit of detection at the beginning of the assay. Therefore the MSC (0.4 µg/L) is estimated based on nominal concentrations, but in reality is likely to be lower still. As cefotaxime is known to be relatively unstable [29] an overnight degradation experiment was conducted to determine the amount of biotic and abiotic degradation occurring in the experimental system. A sterile microcosm and another inoculated with the complex community was incubated and destructively sampled at 0 hours, 6 hours and then every 3 hours for 24 hours. In sterile culture cefotaxime had only partially degraded over 24 hours; whereas in the presence of the community, cefotaxime was undetectable following 12 hours incubation (Figure 4A and 4B). This increase in degradation rate coincided with the beginning of the exponential growth phase of the community (Figure S8).

Extracellular beta-lactamases 'protect' susceptible bacteria at cefotaxime concentrations well above MIC. To confirm this degradation was biotic and by extracellular beta-lactamases, an additional experiment was performed whereby a susceptible *E. coli* strain (J53) was cultured in the presence of supernatant derived from an overnight culture of an *E. coli* strain bearing $bla_{\text{CTX-M}}$-15, $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ on a fully sequenced resistance plasmid [30] (strain NCTC 13451, available from Public Health England). Addition of the supernatant from the resistant strain allowed growth of the susceptible strain at the clinical breakpoint concentration [23], which was over 10x the MIC of the susceptible strain (Figure 5).

DISCUSSION

Here, we quantified positive selection for antibiotic (cefotaxime) resistance in a waste water derived complex bacterial community, by quantifying changes in resistance gene prevalence over time. We show clinically important [31] resistance genes ($bla_{\text{CTX-M}}$) were positively selected at very low, environmentally-relevant concentrations likely due a combination of clonal expansion of hosts carrying $bla_{\text{CTX-M}}$ and horizontal gene transfer of plasmids bearing bla-$_{\text{CTX-M}}$. Antibiotic quantification has been identified as an overlooked aspect of MSC determination [13]. We quantified antibiotic concentrations when determining minimal selective concentrations and found cefotaxime to be rapidly degraded by the community, suggesting the estimated MSC of 0.4 μg/L to be an underestimate. Even so, the cefotaxime MSC determined in this study was very similar to several measured environmental concentrations [25, 32], suggesting selection could occur in certain environments such as hospital effluent and waste water influent. Responses to selection in such environments may be reduced, as unenriched bacterial communities e.g. in sewage may be impacted by high cell

numbers and associated reduction in resource availability. However it is also possible that sustained exposure over long time periods would produce the same response.

In addition, we observed a plateau in the strength of selection across a very large antibiotic concentration range. This novel finding contrasts with previous work which has shown resistance to increase monotonically with antibiotic concentration [3, 5, 14]. A crucial implication of this finding is that selection for clinically important resistance mechanisms, such as $bla_{\text{CTX-M}}$, may occur to a similar extent at sub-inhibitory concentrations as at high, clinical concentrations. The observed plateau in resistance selection has clinical relevance, when considering the antibiotic concentration gradients which inevitably form in different body compartments during chemotherapy [33]. These may also provide greater potential for selection for antibiotic resistance *in vivo* than previously considered. Potential overtreatment with unnecessarily long antibiotic courses [34] may compound this effect. Future research should address this finding and its relevance to environmental protection, effective antibiotic treatment and antimicrobial stewardship.

The observed plateau in selection for resistance is likely due to the cross-protective effect conferred by the resistant fraction on the susceptible fraction of bacteria in the population. Three lines of evidence strongly support this: 1) the degradative effect of the community, whereby within 24 hours all cefotaxime is degraded below the limit of chemical quantification, including the very highest, clinical breakpoint concentration. 2) metagenome analyses of 3 replicates at 4 antibiotic concentrations, which showed the main mechanism of resistance to the treatment antibiotic was degradative in nature, and could therefore provide a benefit to susceptible competitors within the community. 3) The single species *E. coli* experiment, which used supernatant from a resistant strain bearing a multi-resistance plasmid

with the plasmid-free strain to show the potential extent of this community-wide benefit. The level of protection conferred by extracellular beta-lactamases in the supernatant of the resistant strain culture allowed growth of the susceptible strain well above its own MIC, at the clinical breakpoint concentration. Extrapolating this finding to the community, we hypothesise that within each 24 hour time period, CTX-M producers (and possibly other degraders) are selected for by cefotaxime, and are then outcompeted by susceptible bacteria following antibiotic degradation. This means resistant genotypes are likely to persist at even very low antibiotic concentrations, as they provide a benefit to the whole community; this effect has been modelled previously [19].

Selection for *bla*$_{\text{CTX-M}}$ genes is likely due a combination of clonal expansion of hosts carrying *bla*$_{\text{CTX-M}}$ and horizontal gene transfer of plasmids bearing *bla*-$_{\text{CTX-M}}$. Our results are consistent with epidemiological data on beta-lactam resistance genes [35, 36], which document the rapid spread of *bla*$_{\text{CTX-M}}$ genes worldwide to a 'pandemic' status. In this study, *bla*$_{\text{CTX-M}}$ genes were under stronger selection than a large diversity of other resistance genes, possibly due to lower fitness cost (either metabolic or due to genetic context), and/or due to more efficient degradation and a potential wider degradative capacity. For example, previous research found the MICs of *bla*$_{\text{CTX-M}}$ positive bacteria isolated from river sediment downstream of a waste water treatment plant were in excess of 2048 µg/ml [37]. Additionally, many TEM and OXA beta-lactamases do not have the extended-spectrum degradative capability of CTX-M ESBLs [35, 36]. We suggest the ability of *bla*$_{\text{CTX-M}}$ genes to outcompete other beta-lactamase genes at all studied concentrations may also have contributed to the 'pandemic' spread of *bla*$_{\text{CTX-M}}$ genes worldwide [35] and the replacement of other beta-lactamase variants [36].

The metagenome analyses showed *ampC* genes were detected but not enriched by cefotaxime exposure. Overexpression of chromosomal *ampC* genes can increase levels of resistance to many antibiotics, including cefotaxime but these genes were very rare within metagenomes and only confer low level resistance up to 8 mg/L [38] suggesting they do not play a significant role at the community level in this study [39]. The metagenome analyses also showed cefotaxime can also co-select for resistance to a range of antibiotic classes, even at sub-inhibitory cefotaxime concentrations. Co-occurrence of $bla_{\text{CTX-M}}$ genes and aminoglycoside resistance has been reported previously and is confirmed here [36]. High levels of co-selection also indicates presence of multi-drug resistant plasmids, via co-resistance (i.e. co-localisation of multiple genes, of which only one needs to be under positive selection) [40].

The bacterial community analyses showed that cefotaxime had significant effects on community structure, even at sub-inhibitory concentrations through elimination of several species as determined by LEfSe. The bacterial communities in all treatments comprised mainly of Gram negative bacterial families, with *E. coli* being the most abundant species in all treatments. In waste water, with a lower temperature and different nutritional composition, inter-species competition with other taxa could result in reduced *E. coli* abundance which may be favoured by the media and temperature used in this study. However, *E. coli* are known to survive waste water treatment and are used as faecal indicator organisms to assess water quality [41]. Therefore, in waste water, *E. coli* may still possess a competitive advantage. There was much greater variability between replicates (for all species other than *E. coli*) within exposed communities, indicative of potential founder effects on evolution within individual microcosms, again supporting the hypothesis selection was acting at these

291 concentrations. Worryingly, cefotaxime exposure enriched for well-known opportunistic
292 Gram negative pathogens including *P. aeruginosa,* which readily infects
293 immunocompromised individuals such as cystic fibrosis patients; and *A. baumanii,* which is
294 most commonly associated with hospital-acquired infections [42]. Enrichment these
295 opportunistic pathogens and human and gut commensals such as *B. fragilis* and *E. faecalis*
296 under cefotaxime exposure may be due to intrinsic resistance which is likely to result in
297 enrichment within communities including susceptible strains.

298 In summary, our findings develop understanding of selection for antibiotic resistance by
299 showing that the strength of selection for clinically important resistance genes in a community
300 context can be equal across a very large antibiotic concentration gradient; in other words, we
301 show the strength of selection within a given selective space may be constant. Therefore,
302 selection pressure below the MIC of susceptible bacteria may be as strong as selection
303 between the MICs of susceptible and resistant bacteria (traditional selective window). We
304 hypothesise that in our study, this observation is due to the community-wide benefit provided
305 by resistant bacteria harbouring degradative resistance mechanisms. Marked increases in
306 common Gram negative, opportunistic pathogens and co-selection for resistance to other
307 antibiotic classes raises concerns about selection and co-selection for clinically relevant genes
308 (such as $bla_{\text{CTX-M}}$) in pathogenic hosts occurring in a wide range of ecological compartments.

309

310 **MATERIALS AND METHODS**

311 **Complex community collection, storage and preparation.** Domestic sewage influent from a
312 waste water treatment plant serving a small town was collected in October 2015. The treatment

313 plant serves a population of 43 000. Single use aliquots were mixed in a 1:1 ratio with 20 %
314 glycerol, vortexed and stored at -80 °C. Before use, samples were spun down at 21,100 *g* for 10
315 minutes, the supernatant removed, and the pellet resuspended twice in equal volume of 0.85%
316 NaCl to prevent nutrient/chemical carry over.

317 A pilot experiment (data not shown) was conducted to determine the appropriate density of the
318 complex community inoculum by comparing growth over 24hours of different dilutions of
319 inoculum at a range of antibiotic concentrations in a 96 well plate. Following results from the
320 pilot experiment, the 10 % volume/volume waste water inoculum was used for all further
321 experiments using the complex community on the basis it produced the tightest replicates at all
322 the time points and the most reliable growth phases over 24 hours.

323

324 **Selection experiments.** Iso-sensitest broth (Sigma) was inoculated with 10 % volume/volume of
325 untreated waste water. This was separated into 30 ml aliquots and appropriate amounts of
326 cefotaxime stock solution were added. Cefotaxime (Molekular) stocks were prepared in
327 autoclaved and filtered (0.22 µM) deionised water.

328 These 30ml aliquots were further separated into 5ml aliquots, with 5 replicates for each of the
329 cefotaxime assay concentrations: 2 mg/L, 1 mg/L and500, 250, 125, 62.5, 31.25, 15.625 and 0
330 µg/L for the first experiment; and at 128, 64 , 32 , 16 , 8 , 4 , 2 and 0 mg/L for the second
331 experiment.

332 All replicates were immediately sampled for the day 0 sampling time point: 2 x 1ml of each
333 replicate for each treatment was spun down at 21 100 x*g* for 3 minutes, the supernatant removed
334 and pellet resuspended in 1000 µl 20% glycerol followed by storage at -80 °C. All other samples

for DNA extraction were taken following each overnight incubation at 37 °C, 180 rpm shaking, as above.

After each incubation, 50 µl of each microcosm was inoculated into 5ml fresh medium with fresh antibiotic, and samples taken as above for a total of 8 days. Remaining cell suspensions were spun down and stored as above at the end of each experiment.

Metagenome analyses. Three replicates were chosen at random from the no antibiotic, 125 µg/L, 500 µg/L and 2 mg/L treatment to undergo shotgun metagenomic sequencing on the MiSeq2 v2 platform at University of Exeter Sequencing Service (ESS).

DNA was extracted from 1 ml of frozen overnight culture using the MoBio extraction kit according to manufacturer's instructions. DNA was cleaned and concentrated using Ampure™ beads. Firstly, 2 µl of 20 mg/ml RNAse A (Qiagen) was added to 50 µl DNA and incubated for 10 minutes at 37 °C. 50 µl of Ampure™ beads were mixed with the DNA/RNAse solution and mixed gently by pipetting and incubated at room temperature for 5 – 10 minutes. Following pulse centrifugation to collect droplets, tubes were placed on a magnetic stand and left until all beads had precipitated to the side of the tube. Supernatant was removed and beads were washed two times with 300 µl freshly prepared 80 % ethanol. Beads were air dried briefly (1 – 2 minutes), resuspended in 10µl 10mM Tris-HCL and then incubated for another 10 minutes at 50 °C. Following pulse centrifugation and bead precipitation, DNA was transferred into a fresh tube and stored at -20 °C until library preparation and sequencing.

The 12 Nextera Library preparations, quality control, sequencing and primary sequencing analysis (including trimming reads of the barcodes) was performed by ESS. Data was then run

through the "online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured antibiotic resistance gene database", the ARGs-OAP [28]. This provides the abundance of different resistance gene classes and subtypes within these groups normalised by parts per million, 16S rRNA copy number, and cell number. For all subsequent analysis, data normalised by 16S rRNA copy number was used to be in accordance with the qPCR data generated. Heatmaps for resistance gene abundance were generated using pandas [43], matplotlib [44] and seaborn [45] Python packages for resistance gene class and beta-lactam resistance gene subtype, for averages of these three replicates.

16S rRNA sequences were extracted from the Illumina sequencing data as follows: first, forward and reverse reads were adaptor trimmed using Skewer [46] in paired end mode. Fastqc [47] and Multiqc [48] verified successful adaptor removal and that sequences were of acceptable quality before paired end reads were combined with FLASH version 2 [49] with the maximum overlap set to 300. 16S rRNA sequences were extracted and assigned to bacterial species using Metaphlan2 [27]. The resulting heatmap of species relative abundance was generated using HClust2 using Bray Curtis distance measurements between samples and features (species) [50]; and overall species abundance across treatments was visualised with GraPhlan [51]. Linear Discriminant Analyses (LDA) effect size (LEfSe) analyses was performed and visualised with LEfSe [52] to identify which species, if any, were enriched by a particular cefotaxime treatment.

qPCR analyses. Frozen samples/were thawed and DNA extracted using the MBio UltraClean DNA extraction Kit according to instructions. DNA was diluted 5 – 10x before use.

The qPCR conditions were optimised using primers from previous studies [53, 54]: 10 µl Brilliant qPCR SYBR Green master mix, 2 µl primer pair (5 µM each of forward and reverse primers for 16S rRNA, and 9 µM each of forward and reverse CTX-M primers), 0.2 µl BSA (20 mg/ml), 0.4 µl ROX reference dye (20 µM), 5 µl diluted DNA template and filtered, sterilised water to a total volume of 20 µl. The qPCR programme for all reactions was 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. $Bla_{\text{CTX-M}}$ copy number was divided by 16S rRNA copy number to determine $bla_{\text{CTX-M}}$ gene/16S rRNA, a proxy for $bla_{\text{CTX-M}}$ prevalence.

gBlock synthetic genes (IDTDNA - see Table 1) were used as qPCR standards; these were resuspended in TE buffer according to the manufacturer's instructions and were stored at -80 °C.

Standards were 10 x serially diluted in TE buffer and stored at -20 °C before use. Every PCR plate was always run with 5 serial dilutions of standards in duplicate (and a duplicate negative control). Provided the efficiency for the reaction was between 90 % and 110 %, the average CT's for the duplicate technical replicates for each sample was used to calculate the copy number based on a 'gold standard' standard series, where the DNA concentration had been quantified by QuBit and the copy number per µl quantified immediately prior to cycling.

Data analyses. All statistics were performed in R Studio [55]. Selection coefficients were used to determine MSC, with the following equation, as previously[3]: $[\ln(R(t)/R(0))]/[t]$, where R = resistance prevalence, t = time in days and R(0) = resistance prevalence at time zero. The MSC is estimated by the line of best fit X-axis intersect. Figures were generated with various python packages [43-45].

Chemical extraction/analyses. Complex community microcosms were sampled at the Day 0 time point and after the first 24 hours. Antibiotic stocks were also quantified.

The extraction procedure was as follows: 400 µl of culture was mixed with 400 µl HiPerChromosolv Acetonitrile in a 2 ml 96 well plate, and spun at 3500 rpm for 30 minutes. 100 µl of this supernatant was then mixed with 900 µl of 1:4 Acetonitrile to HPLC-grade water in a fresh plate, and stored in the fridge. Antibiotic stocks were diluted to a final concentration of 100 ng/L in 1:4 Acetonitrile. Extractions were kept at 4 °C until processing.

Each concentration from the evolution experiment had a minimum of two chemical replicates from at least two of the biological replicates. Stocks were single replicate only.

Chemical quantification was performed at the University of Exeter Streatham Campus by Maciek Trnzadel and Malcolm Hetherdige, co-funded by AstraZeneca Global SHE and the University of Exeter.

Antibiotic degradation experiment. Washed, untreated waste water was diluted in 25ml Iso-sensitest broth aliquots spiked with cefotaxime concentrations of 0, 15.625, 31.25, 62.5, 125, 250, 500 ug/L or 2 mg/L. A sterile control at the same concentrations was also prepared. These were incubated at 37 °C, 180 rpm shaking in between sampling. Chemical extractions (see below) and destructive sampling for OD readings were performed at time 0, then every 3 hours for 24 hours. OD measurements were carried out in a spectrophotometer (Jenway, UK) at the same time points at 600 nm. Any OD readings with a value greater than 1 were diluted 10 x in Iso-sensitest broth and then re-measured.

Supernatant experiment. *E.coli* strains J53 and NCTC 13451 were grown overnight at 37 °C, shaking at 180 rpm in Iso-sensitest broth (supplemented with 2 mg/L cefotaxime for NCTC 13451). This concentration was chosen on the basis it was greater than the J53 MIC (> 250 µg/L and < 500 µg/L, determined by microdilution assay [56]), that it would be fully degraded in a beta-lactamase producing community (according to the overnight degradation experiment); and because it is the clinical breakpoint concentration for *Enterobacteriaceae* [23]. The supernatants from both overnight cultures were spun down at 21 000 *x g* for 2 minutes twice, and then filtered through a 0.22 µM filter. J53 was inoculated at a starting optical density (600nm) of 0.01 into fresh Iso-sensitest broth amended with 2 mg/L and J53 or NCTC 13451 supernatant (12.5% volume/volume). Controls included a blank control (to check general aseptic technique), broth with each supernatant (to verify the supernatant was sterile); and J53 in broth both with and without antibiotic (to deduce effects of nutrient dilution).

ACKNOWLEDGEMENTS

Aimee Murray was supported by a BBSRC/AZ CASE Studentship, BB/L502509/1. Lihong Zhang was supported Natural Environment Research Council grant NE/M01133X/1. Chemical extraction and quantification protocols, and chemical quantification were performed by Maciej Trznadel and Malcolm Hetheridge, University of Exeter and were funded by AstraZeneca,

REFERENCES

1. O'Neill, *O'Neill Report Wellcome Trust. Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations.* 2014.

2. O'Neill, *Review on Antimicrobial Resistance: Tackling Drug-Resistant Infections Globally. Antimicrobials in agriculture and the environment: reducing unnecessary use and waste.*, H. Government, Editor. 2015.

3. Gullberg, E., et al., *Selection of Resistant Bacteria at Very Low Antibiotic Concentrations.* Plos Pathogens, 2011. **7**(7).

4. Andersson, D.I. and D. Hughes, *Evolution of antibiotic resistance at non-lethal drug concentrations.* Drug Resistance Updates, 2012. **15**(3): p. 162-172.

5. Gullberg, E., et al., *Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals.* MBio, 2014. **5**.

6. Negri, M.C., et al., *Concentration-dependent selection of small phenotypic differences in TEM beta-lactamase-mediated antibiotic resistance.* Antimicrobial Agents and Chemotherapy, 2000. **44**(9): p. 2485-2491.

7. Negri, M.-C., et al., *Very Low Cefotaxime Concentrations Select for Hypermutable Streptococcus pneumoniae Populations.* Antimicrobial Agents and Chemotherapy, 2002. **46**(2): p. 528-530.

8. Bengtsson-Palme, J. and D.G. Larsson, *Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation.* Environ Int, 2016. **86**: p. 140-9.

9. Baquero, F. and T.M. Coque, *Widening the spaces of selection: evolution along sublethal antimicrobial gradients.* MBio, 2014. **5**(6): p. e02270.

10. Volkmann, H., et al., *Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan).* Journal of Microbiological Methods, 2004. **56**(2): p. 277-286.

11. Schwartz, T., et al., *Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms.* Fems Microbiology Ecology, 2003. **43**(3): p. 325-335.

12. Hultman, J., et al., *Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent.* FEMS Microbiol Ecol, 2018. **94**(4).

13. Karkman, A., et al., *Antibiotic-Resistance Genes in Waste Water.* Trends in Microbiology, 2018. **26**(3): p. 220-228.

14. Lundstrom, S.V., et al., *Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms.* Sci Total Environ, 2016. **553**: p. 587-595.

15. WHO, *Critically Important Antimicrobials for Human Medicine*. 2011, Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR).

16. WHO, *19th WHO Model List of Essential Medicines*. 2015.

17. WHO, *WHO Model List of ssential Medicines*. 2017.

18. Livermore, D.M., *beta-Lactamases in laboratory and clinical resistance.* Clinical Microbiology Reviews, 1995. **8**(4): p. 557-584.

19. Dugatkin, L.A., M. Perlin, and R. Atlas, *The Evolution of Group-beneficial Traits in the Absence of Between-group Selection.* Journal of Theoretical Biology, 2003. **220**(1): p. 67-74.

20. Medaney, F., et al., *Live to cheat another day: bacterial dormancy facilitates the social exploitation of beta-lactamases.* ISME J, 2015.

21. Yurtsev, E.A., et al., *Bacterial cheating drives the population dynamics of cooperative antibiotic resistance plasmids.* Molecular Systems Biology, 2013. **9**(1).

22. Bottery, M.J., A.J. Wood, and M.A. Brockhurst, *Selective Conditions for a Multidrug Resistance Plasmid Depend on the Sociality of Antibiotic Resistance.* Antimicrob Agents Chemother, 2016. **60**(4): p. 2524-7.

23. EUCAST, *The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. .* 2014. **Version 4.0**.

24. Gomez, M.J., et al., *Solid-phase extraction followed by liquid chromatography-time-of-flight-mass spectrometry to evaluate pharmaceuticals in effluents. A pilot monitoring study.* J Environ Monit, 2007. **9**(7): p. 718-29.

25. UmweltBundesamt, *Pharmaceuticals in the environment*, UBA, Editor. 2016.

26. Homem, V. and L. Santos, *Degradation and removal methods of antibiotics from aqueous matrices - A review.* Journal of Environmental Management, 2011. **92**(10): p. 2304-2347.

505 27. Truong, D.T., et al., *MetaPhlAn2 for enhanced metagenomic taxonomic profiling.* Nat Methods, 2015. **12**(10): p. 902-3.

507 28. Yang, Y., et al., *ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database.* Bioinformatics, 2016. **32**(15): p. 2346-51.

510 29. Das Gupta, V., *Stability of cefotaxime sodium as determined by high-performance liquid chromatography.* J Pharm Sci, 1984. **73**(4): p. 565-7.

512 30. Woodford, N., et al., *Complete Nucleotide Sequences of Plasmids pEK204, pEK499, and pEK516, Encoding CTX-M Enzymes in Three Major Escherichia coli Lineages from the United Kingdom, All Belonging to the International O25:H4-ST131 Clone.* Antimicrobial Agents and Chemotherapy, 2009. **53**(10): p. 4472-4482.

517 31. CDC, *Antibiotic resistance threats in the United States, 2013*. 2013, Centre for Disease Control and Prevention.

519 32. Gulkowska, A., et al., *Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China.* Water Research, 2008. **42**(1–2): p. 395-403.

522 33. Pena-Miller, R., et al., *When the most potent combination of antibiotics selects for the greatest bacterial load: the smile-frown transition.* PLoS Biol, 2013. **11**(4): p. e1001540.

525 34. Llewelyn, M.J., et al., *The antibiotic course has had its day.* BMJ, 2017. **358**.

526 35. Canton, R. and T.M. Coque, *The CTX-M beta-lactamase pandemic.* Current
527 Opinion in Microbiology, 2006. **9**(5): p. 466-475.
528 36. Cantón, R., J.M. González-Alba, and J.C. Galán, *CTX-M Enzymes: Origin and*
529 *Diffusion.* Frontiers in Microbiology, 2012. **3**: p. 110.
530 37. Amos, G.C., et al., *Waste water effluent contributes to the dissemination of CTX-*
531 *M-15 in the natural environment.* J Antimicrob Chemother, 2014.
532 38. Peter-Getzlaff, S., et al., *Detection of AmpC Beta-Lactamase in Escherichia coli:*
533 *Comparison of Three Phenotypic Confirmation Assays and Genetic Analysis.*
534 Journal of Clinical Microbiology, 2011. **49**(8): p. 2924-2932.
535 39. Jacoby, G.A., *AmpC beta-lactamases.* Clin Microbiol Rev, 2009. **22**(1): p. 161-82,
536 Table of Contents.
537 40. Baker-Austin, C., et al., *Co-selection of antibiotic and metal resistance.* Trends in
538 Microbiology, 2006. **14**(4): p. 176-182.
539 41. Ashbolt, N.J., W.O.K. Grabow, and M. Snozzi, *Indicators of microbial water*
540 *quality*, in *Water Quality: Guidelines, Standards and Health*, L. Fewtrell and J.
541 Bartram, Editors. 2001, WHO: London.
542 42. Howard, A., et al., *Acinetobacter baumannii: An emerging opportunistic*
543 *pathogen.* Virulence, 2012. **3**(3): p. 243-250.
544 43. McKinney, W., *Data structures for Statistical Computing in Python*. Proceedings
545 of the 9th Python in Science Conference. 2010.
546 44. Hunter, J.D., *Matplotlib: A 2D graphics environment.* Computing In Science &
547 Engineering, 2007. **9**(3): p. 90-95.

548 45. Waskom, M., *seaborn: v0.7.1 (June 2016)*. 2016.

549 46. Jiang, H., et al., *Skewer: a fast and accurate adapter trimmer for next-generation*
550 *sequencing paired-end reads.* BMC Bioinformatics, 2014. **15**(1): p. 182.

551 47. Andrews, S., *FastQC A Quality Control tool for High Throughput Sequence Data*.

552 48. Ewels, P., et al., *MultiQC: summarize analysis results for multiple tools and*
553 *samples in a single report.* Bioinformatics, 2016. **32**(19): p. 3047-3048.

554 49. Magoc, T. and S.L. Salzberg, *FLASH: fast length adjustment of short reads to*
555 *improve genome assemblies.* Bioinformatics, 2011. **27**(21): p. 2957-63.

556 50. Segata, N., *HClust2.*

557 51. Asnicar, F., et al., *Compact graphical representation of phylogenetic data and*
558 *metadata with GraPhlAn.* PeerJ, 2015. **3**: p. e1029.

559 52. Segata, N., et al., *Metagenomic biomarker discovery and explanation.* Genome
560 Biol, 2011. **12**(6): p. R60.

561 53. Birkett, C.I., et al., *Real-time TaqMan PCR for rapid detection and typing of genes*
562 *encoding CTX-M extended-spectrum β-lactamases.* Journal of Medical
563 Microbiology, 2007. **56**(1): p. 52-55.

564 54. Czekalski, N., E. Gascon Diez, and H. Burgmann, *Wastewater as a point source of*
565 *antibiotic-resistance genes in the sediment of a freshwater lake.* ISME J, 2014.

566 55. RStudio, *RStudio: Integrated Development for R. RStudio, Inc., Boston, MA.* 2015.

567 56. Andrews, J., *Determination of minimum inhibitory concentrations.* Journal of
568 Antimicrobial Chemotherapy, 2001. **48**(S1): p. 15-16.

569

570 **TABLES**

571 Table 1. List of synthetic gene blocks used in the study, their DNA sequence, size in bp, and

572 accession numbers used to for design.

Gene	Sequence	Size (bp)	Accession numbers
16S	ACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCG	144	CP018770.2
CTX-M	GATGTGCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCGACAGCTGGGAGACGAAACGTTCC	338	KX452391.1

	GTCTCGACCGTACCGAGCCGACGTTAAAC ACCGCCATTCCGGGCGATCCGCGTGATA		

573

574 **FIGURE LEGENDS**

575 Figure 1. Heatmap showing the relative abundance of detected species using Bray Curtis distance
576 measurements for treatment (x axis) and species (y axis) for each cefotaxime treatment. 'C0',
577 'C4', 'C6' and 'C8' correspond to 0, 125, 500 and 2000 µg/L cefotaxime respectively. The
578 number after the concentration denotes the biological replicate number (1 – 5), chosen randomly
579 for sequencing at day 8 of the experiment.

580

581 Figure 2. Heatmap showing average (n=3) detected beta-lactam resistance gene subtype relative
582 abundance (resistance gene number normalised with 16S rRNA copy number), following 8 days
583 culture with cefotaxime. Only genes detected with the ARGs-OAP pipeline are shown.

584

585 Figure 3. Selection coefficients (n=5) for each cefotaxime concentration, which equal the natural
586 log of resistance gene prevalence ($bla_{CTX\text{-}M}$ gene/16S rRNA copy number) at day 0 and day 8. ●
587 = selection coefficients from low concentration experiment and ■ = from high concentration
588 experiment. Selection coefficients > 0 indicate positive selection.

589

590 Figure 4. Single biological replicate, duplicate chemical replicate chemical quantification of
591 cefotaxime ('Measured cefotaxime concentration ug/L) at 0 and 6 hours, then every 3 hours for

24 hours at different starting cefotaxime concentrations (ug/L) in the presence of the complex bacterial community (A) and in sterile culture (B).

Figure 5. Average (n=4) optical density (600nm) over time of susceptible *E. coli* strain J53 grown in 2000 µg/L cefotaxime (clinical breakpoint concentration for *Enterobacteriaceae*) with beta-lactamase containing supernatant (NCTC strain 13451) or beta-lactamase free supernatant (strain J53).

SUPPLEMENTARY LEGENDS

Figure S1. Heatmap showing relative abundance of all detected species using Bray Curtis distance measurements for treatment (x axis) and species (y axis) for each cefotaxime treatment. 'C0', 'C4', 'C6' and 'C8' correspond to 0, 125, 500 and 2000 µg/L cefotaxime respectively. The number after the concentration denotes the biological replicate number (1 – 5), chosen randomly for sequencing at day 8 of the experiment.

Figure S2. Linear Discriminant Analyses (LDA) effect size (LEfSe) analyses of statistically significant species associated with different cefotaxime treatments. Negative LDA scores (red) show species enriched in the no antibiotic treatment, and positive LDA scores (green) show species enriched in the 2000 µg/L cefotaxime treatment.

Figure S3. Heatmap showing average (biological replicate n=3) resistance gene relative abundance (resistance gene number normalised with 16S rRNA copy number), following 8 days culture with cefotaxime. “Macrolide-Linco-Strept” = Macrolide, Lincosamide and Streptogramin resistance.

Figure S4. MSC (cefotaxime concentration at the x-axis intercept) determination using average (n=5) selection coefficients (natural log of *bla*$_{\text{CTX-M}}$ prevalence over 8 days, *bla*$_{\text{CTX-M}}$ prevalence = *bla*$_{\text{CTX-M}}$ copy number/16S rRNA copy number, qPCR technical replicate n=2). Shown with standard error bars (of biological replicates) and polynomial (order 2) line of best fit.

Figure S5. Average (biological replicate n=5, technical qPCR replicate of each biological replicate n=2) bla$_{\text{CTX-M}}$ prevalence (bla$_{\text{CTX-M}}$ copy number/16S rRNA copy number) number at day 0 and following 1, 4 and 8 days of cefotaxime exposure. Shown with standard error bars (of biological replicates).

Figure S6. Average (biological replicate n=5, technical qPCR replicate of each biological replicate n=2) bla$_{\text{CTX-M}}$ copy number following 8 days cefotaxime exposure in the higher concentration experiment. Shown with standard error bars (of biological replicates).

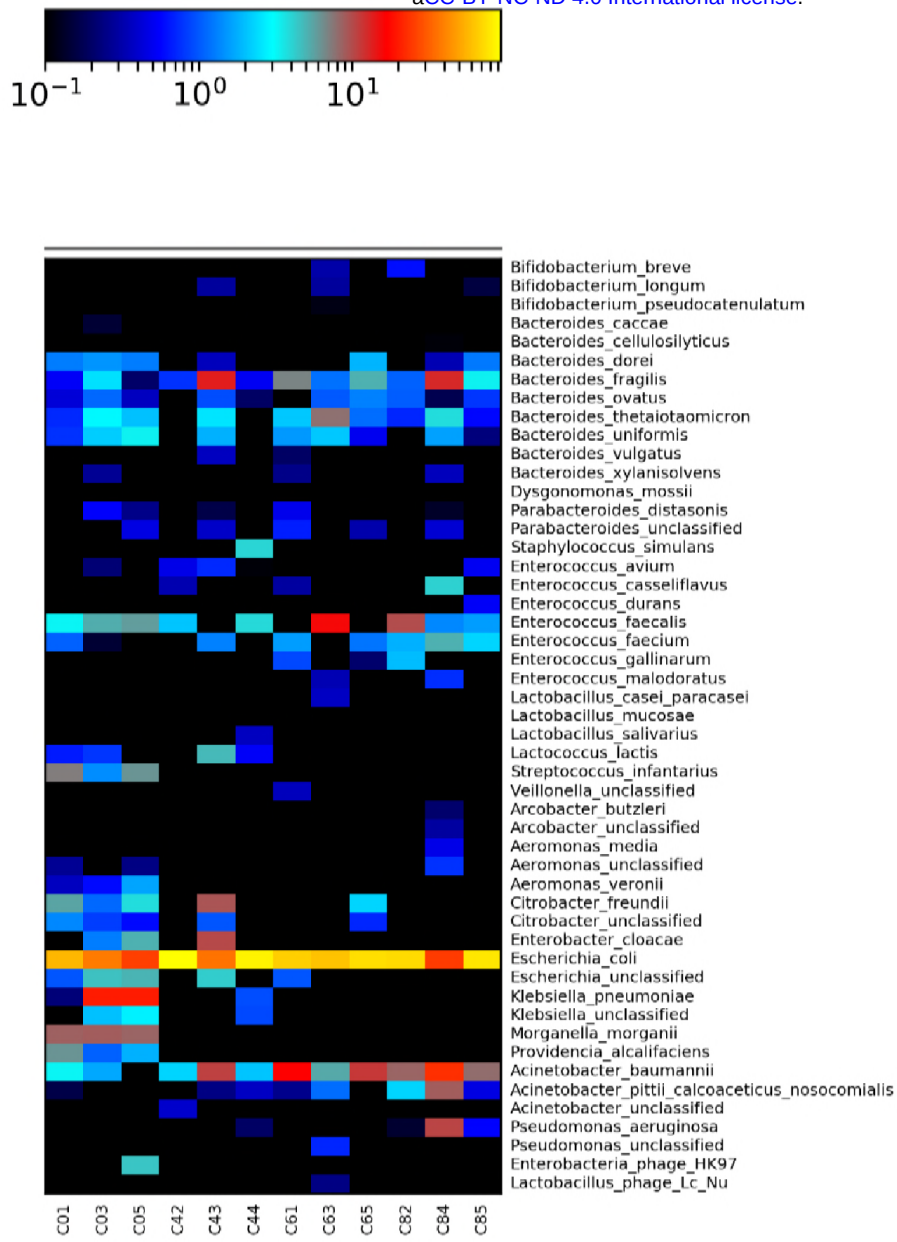
631 Figure S7. Average (biological replicate n=5, technical qPCR replicate of each biological
632 replicate n=2) 16S rRNA copy number following 8 days cefotaxime exposure, in the higher
633 concentration experiment. Shown with standard error bars (of biological replicates).

634

635 Figure S8. Growth (optical density (600nm)) of the complex community over time during the 24
636 hour degradation experiment. Single replicate only.

637

638 Table S1. Nominal (expected) and average (biological replicate n=3, technical replicate of each
639 n=2) measured cefotaxime concentrations as determined by LC-MS at the beginning (time 0) of
640 the selection experiment, and after 24 hours culture at 180rpm, 37°C in the presence of the
641 complex community. Also shown are the cefotaxime stocks (‘1’ and ‘2’) used in the experiment.



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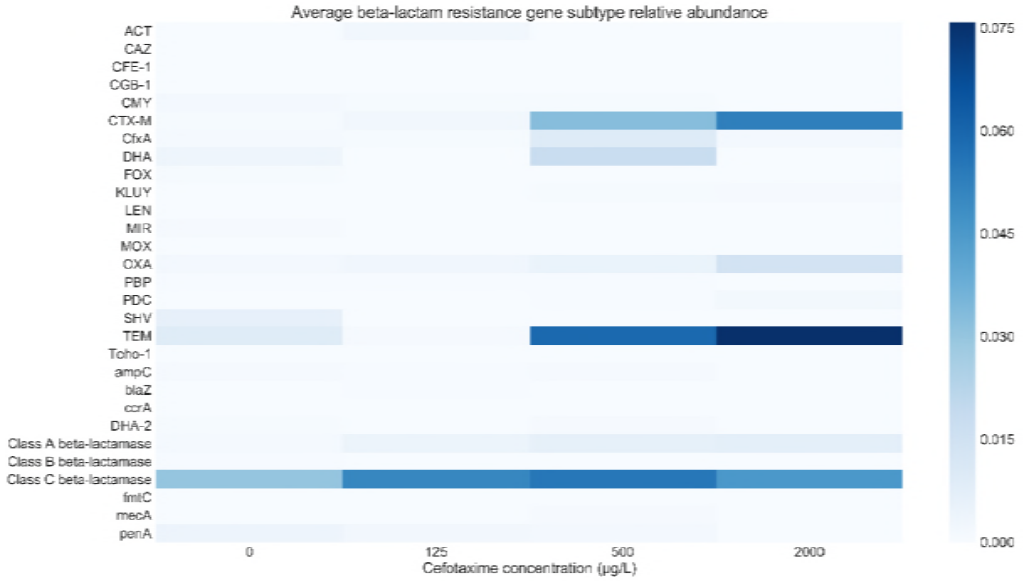

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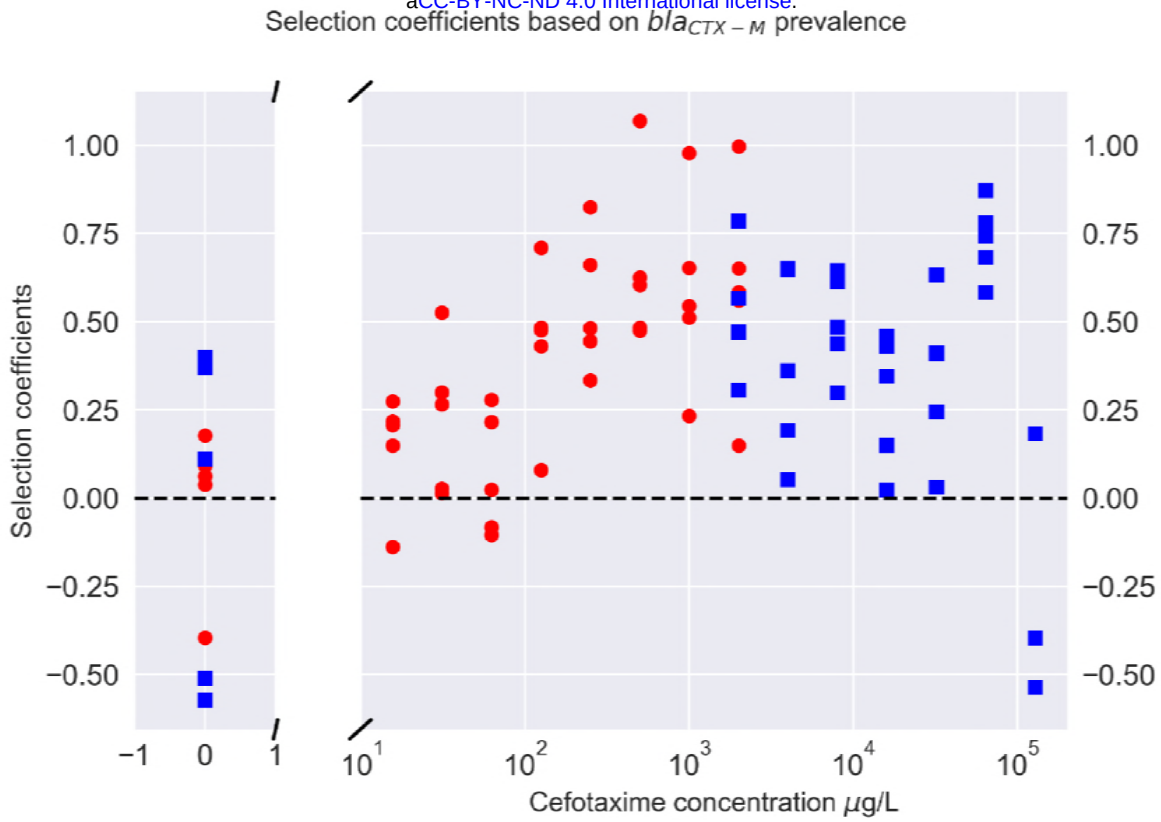

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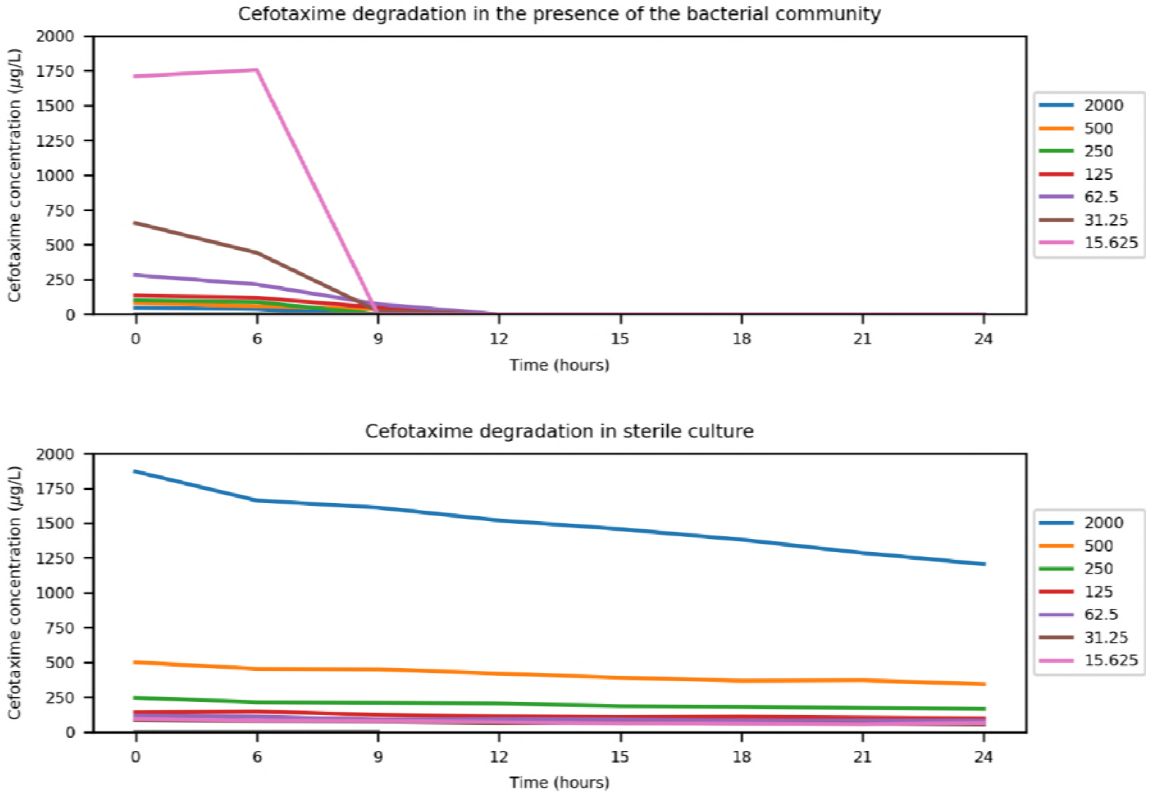

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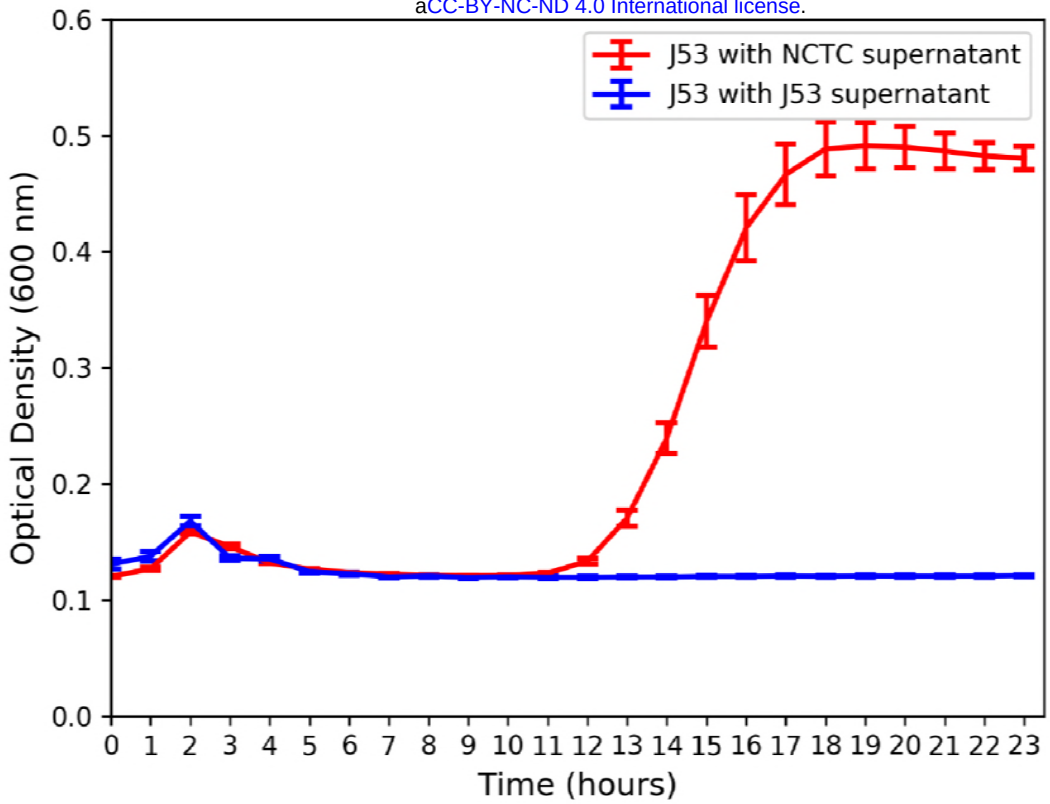

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