Why Limit Antibiotic Pollution? The Role of Environmental Selection in Antibiotic Resistance Development

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Antibiotic resistance is estimated to cause hundreds of thousands of deaths every year.[1] At the same time, new types of antibiotic resistance mechanisms continue to be discovered among both pathogenic and harmless bacteria.[2,3] This development points towards the existence of a large source of resistance factors outside of the human microbiome – the environmental bacteria present virtually everywhere on earth.[4,5] Although many resistance genes already have emerged in pathogens, their specific origin and the circumstances that favored their transition are still largely unclear.[6] Most likely, the most critical factor in the emergence, mobilization and spread of novel resistance genes to human and animal pathogens is a selection pressure from antibiotics.[7] Bacteria in the human and animal gut flora are frequently exposed to sufficiently high concentrations of antibiotics to select for resistant strains. However, an often overlooked aspect is that the taxonomic diversity of the gut flora, and hence the available source of potential novel resistance factors, is tiny compared to that of environmental microbial communities. Research over the past decades indicates that antibiotic residues also reach the environment and, in some cases, select for resistance. Therefore, a fundamental understanding of where selective conditions for antibiotic resistance exist is crucial in order to develop comprehensive mitigation strategies that will avoid or delay future resistance development associated with the environmental resistome.[8]

Defining selective concentrations of antibiotics

Antibiotics can exert selection for resistance at concentrations below those that completely inhibit bacterial growth.[9] To identify environments that may act as spawning grounds for resistance, we must define the concentrations of antibiotics that can drive resistance development. Several approaches have been explored to determine these minimal selective concentrations,[7] ranging from biologically simplistic, but very precise competition experiments between resistant and non-resistant strains,[9] to experimental setups aimed at capturing the complex interplay of full-scale microbial communities.[10] From an ecological standpoint, methods designed to quantify the levels

Box 1. Predicting "no-effect concentrations" for antibiotics

- The lowest minimal inhibitory concentration (MIC) for each species-antibiotic combination in EUCAST was collected. For some antibiotics, the number of tested species was small.
- The potential bias caused by low species coverage on the observed lowest MICs was estimated by sub-sampling MIC data for the antibiotics with many tested species. This sub-sampling data was used to predict size-adjusted lowest MICs.
- An assessment factor of 10 was then applied to account for that minimal selective concentrations by necessity would be lower than the MICs, arriving at predicted "no-effect concentrations" (PNECs) for 111 antibiotics in total.

Note: MICs and PNECs in Table 1 are rounded down to the closest number on the EUCAST testing scale. We also compared each PNEC to the highest concentration reported in effluents from sewage treatment plants.[15]

of an antibiotic that promote resistance in complex communities should better capture the effects of sub-inhibitory concentrations found in real environments, such as wastewater treatment plants and their recipients. However, both competition experiments and microcosm investigations of complex communities are labor-intensive. Nonetheless, a reference framework for selective concentrations of antibiotics is urgently needed.[11] Therefore, we took advantage of the EUCAST database[12] containing minimal inhibitory concentrations for a large range of bacteria (170 species) and used it to predict the estimated "no-effect" concentrations for 111 antibiotics (Box 1; Table 1).[13] The estimated "no-effect" concentrations are based on the assumption that an antibiotic concentration that inhibits growth of certain bacterial species will also be selective, since it enables non-susceptible strains to outcompete sensitive ones – at least in some communities. The predicted no-effect concentration for tetracycline has subsequently been validated experimentally in complex aquatic biofilms and shown to be in the expected range[10] (Figure 1). Many of the predicted no-effect concentrations for resistance selection are substantially below those expected to have ecotoxicological effects on other organisms.[13]

Identifying environments at risk for selection of resistance factors

With a framework of no-effect concentrations for resistance selection in place, we are now able to identify environments that bear the potential to confront bacteria with selective conditions. One environment that often has been suggested as a "hotspot" for resistance development is the sewage treatment plant.[14] When we apply the above framework, we can see that measured concentrations of antibiotics in untreated sewage influent often barely attain predicted no-effect concentrations, and only do so for a few antibiotics.[13,15] Furthermore, in a recent study of ours where concentrations of ciprofloxacin and tetracycline in influent water were slightly

Table 1. Estimated minimal selection concentration boundaries and predicted no-effect concentrations for 26 commonly used antibiotics

Antibiotic	Antibiotic class	N[1]	Covered genera (famlies)	Observed lowest MIC (µg/L)	Size-adjusted predicted lowest MIC (µg/L)[2]	PNEC (incl. assessment factor) (µg/L)	STP Effluent conc. (µg/L)[3]
Gentamicin	Aminoglycosides	68	27 (14)	16	16	1	1.3
Tobramycin	Aminoglycosides	31	15 (8)	16	8	1	
Co-trimoxazole	Antifolate combinations	36	22 (13)	8	4	0.5	
Ertapenem	Carbapenems	36	20 (12)	2	1	0.125	
Cefalexin	Cephalosporins (1st gen.)	10	7 (5)	250	32	4	1.8
Cefaclor	Cephalosporins (2nd gen.)	11	7 (6)	32	8	0.5	1.8
Cefdinir	Cephalosporins (3rd gen.)	5	4 (4)	32	2	0.25	
Benzylpenicillin (G)	Narrow-spectrum penicillins	47	12 (11)	4	4	0.25	
Phenoxymethylpenicillin (V)	Narrow-spectrum penicillins	8	5 (5)	4	0.5	0.064	2
Amoxicillin	Extended spectrum penicillins	29	19 (12)	4	2	0.25	0.05
Ampicillin	Extended spectrum penicillins	64	25 (15)	4	4	0.25	0.126
Vancomycin	Glycopeptides	42	10 (9)	125	125	8	0.04
Daptomycin	Lipopeptide	16	6 (6)	32	8	1	
Azithromycin	Macrolides	12	6 (6)	16	4	0.25	0.38
Clarithromycin	Macrolides	15	10 (10)	8	2	0.25	0.61
Erythromycin	Macrolides	39	14 (13)	16	8	1	0.62
Linezolid	Oxazolidinones	29	9 (9)	125	64	8	
Chloramphenicol	Amphenicols	29	18 (11)	125	64	8	
Colistin	Polypeptides	16	10 (4)	64	16	2	
Ciprofloxacin	Fluoroquinolones (2nd gen.)	70	29 (18)	2	1	0.064	0.742
Levofloxacin	Fluoroquinolones (3rd gen.)	43	24 (16)	4	4	0.25	
Moxifloxacin	Fluoroquinolones (4th gen.)	53	21 (14)	2	2	0.125	0.017
Rifampicin	Rifamycins	19	12 (12)	2	0.5	0.064	
Tigecycline	Glycylcyclines	54	26 (16)	16	16	1	
Doxycycline	Tetracyclines	29	20 (11)	32	16	2	0.915
Tetracycline	Tetracyclines	66	30 (18)	16	16	1	0.62

Notes: 1. These numbers correspond to the number of different species present in EUCAST that could be matched to a valid species name in the SILVA database. 2. The size-adjusted predicted lowest MIC correspond to the estimated upper boundary for the minimal selective concentrations. 3. The highest concentration observed in effluents from conventional sewage treatment plants, as reported by Michael et al.[15]

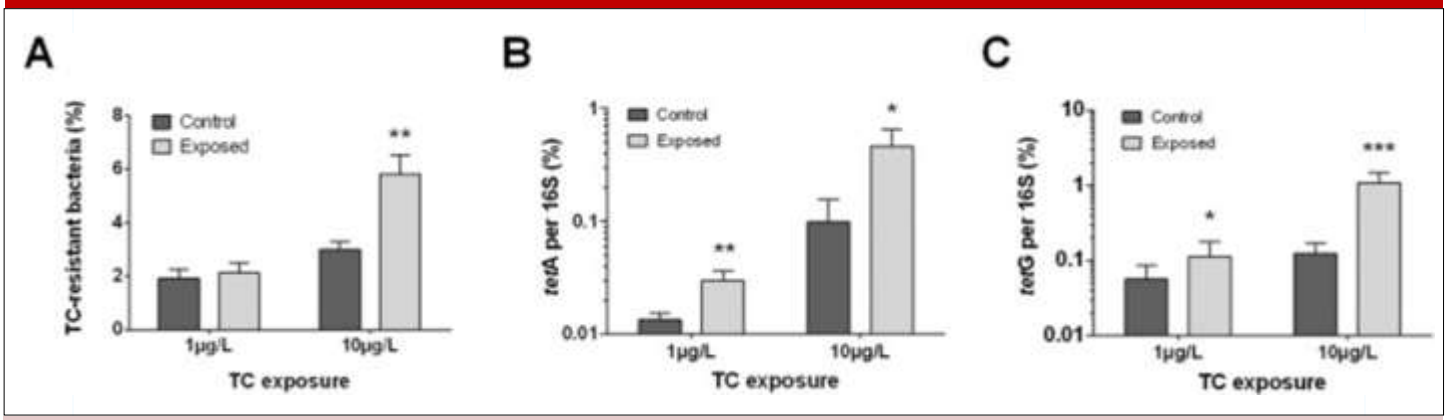
above the predicted no-effect concentrations, no consistent enrichment of genes encoding resistance against any class of antibiotics was observed during the treatment process.[16] Taken together, the evidence for resistance selection in sewage treatment plants is still limited, but at the same time, many of the studies, including ours, have shortcomings that limit interpretation. This is partly because we know little about how mixtures of antibiotics act, but also because of the immense changes in species composition that occur in sewage treatment plants due to various other factors that may mask the effects of direct antibiotic selection. More comprehensive culture-based studies on changes in resistant/non-resistant strains within species are therefore required, both in sewage treatment plants and in receiving waters.

The conditions in sewage treatment plants can be contrasted with those in environments that are subjected to pollution from antibiotic manufacturing. In the latter, substantially higher concentrations of antibiotics have repeatedly been measured,[17] sometimes greatly exceeding the therapeutic concentrations found in human blood during treatment.[18] In several instances, this has been associated with high abundance of resistance genes and – perhaps even more worryingly – a vast diversity of resistance mechanisms, along with genes responsible for horizontal gene transfer.[19,20]

Developing mitigation strategies

The WHO acknowledges that mitigations to limit resistance development should employ a One-Health approach that also includes the external environment.[21] It seems reasonable that priority should be given to measures that would be relatively straightforward to enforce, are associated with limited cost, and carry a large potential impact. Accordingly, the recent O'Neill review on antimicrobial resistance[1] highlights the urgent need to take control of antibiotic pollution from manufacturing, beginning with the discharge limits we have published.[13] Such discharge limits could be applied not only in the form of local regulations, but also during procurement by major buyers of antimicrobials.[22] We also believe there is a need for action in environments contaminated by antibiotic residues from both animal farming and human habitation.[23] However, the overall risks associated with transmission of already-resistant pathogens may very well exceed the risk associated with residues of selective agents in these environments. Both of these different risk scenarios are important to consider when taking actions to manage

Figure 1. Minimal selective concentrations for tetracycline



Results of two experiments with tetracycline (TC; 1 and 10 µg/L versus matched controls) aiming at determining minimal selective concentrations for both phenotypic and genotypic resistance endpoints. (A) Percent TC-resistant bacteria as determined by comparing the number of colony forming units on R2A plates with or without TC (20 µg/mL). A significant increase was demonstrated for 10 µg/L ($p = 0.0045$) but not for 1 µg/L ($p = 0.34$). (B) Relative changes in *tetA* levels as determined by quantitative PCR (1 µg/L, $p = 0.005$; 10 µg/L, $p = 0.017$) (C) Relative changes in *tetG* levels as determined by quantitative PCR (1 µg/L, $p = 0.026$; 10 µg/L, $p = 9.95 \times 10^{-7}$). One-tailed Student's t-tests were performed using percentages (A) or the log2 values of the relative difference in gene-levels between *tet*-genes and 16S rRNA (B,C). Reprinted from Science of the Total Environment,[10] copyright (2016), with permission from Elsevier.

discharges. While installation of advanced sewage treatment technologies may certainly be warranted in specific situations, more is likely to be gained globally in terms of reduced resistance risks by implementing basic sewage treatment systems in low-income regions of the world.[24]

Some knowledge gaps to address

The predicted no-effect concentrations for antibiotic resistance selection are not thought to be set in stone.[13] Instead, they should ideally be complemented with experimental data as it becomes available. Furthermore, other effects of sub-lethal antibiotic levels, e.g., those exerted on horizontal gene transfer,[25,26] are only starting to be elucidated. We also know little about the potential contribution of co-selective agents, such as biocides and metals, on antibiotic resistance development,[27] or how mixtures of antibiotics should be assessed.[28] Still, the urgency of addressing the accelerating antibiotic resistance threat makes it overly clear that we cannot let these knowledge gaps delay the initiation of relevant mitigation efforts in areas where improvements can be made relatively easily.

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