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Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation

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ABSTRACT

There are concerns that selection pressure from antibiotics in the environment may accelerate the evolution and dissemination of antibiotic-resistant pathogens. Nevertheless, there is currently no regulatory system that takes such risks into account. In part, this is due to limited knowledge of environmental concentrations that might exert selection for resistant bacteria. To experimentally determine minimal selective concentrations in complex microbial ecosystems for all antibiotics would involve considerable effort. In this work, our aim was to estimate upper boundaries for selective concentrations for all common antibiotics, based on the assumption that selective concentrations a priori need to be lower than those completely inhibiting growth. Data on Minimal Inhibitory Concentrations (MICs) were obtained for 111 antibiotics from the public EUCAST database. The 1% lowest observed MICs were identified, and to compensate for limited species coverage, predicted lowest MICs adjusted for the number of tested species were extrapolated through modeling. Predicted No Effect Concentrations (PNECs) for resistance selection were then assessed using an assessment factor of 10 to account for differences between MICs and minimal selective concentrations. The resulting PNECs ranged from 8 ng/L to 64 μg/L. Furthermore, the link between taxonomic similarity between species and lowest MIC was weak. This work provides estimated upper boundaries for selective concentrations (lowest MICs) and PNECs for resistance selection for all common antibiotics. In most cases, PNECs for selection of resistance were below available PNECs for ecotoxicological effects. The generated PNECs can guide implementation of compound-specific emission limits that take into account risks for resistance promotion.

1. Introduction

Antibiotic resistance has in the last decades put an increasing pressure on human healthcare globally, estimated to account for 700,000 deaths every year (Review on Antimicrobial Resistance, 2014). The environment has repeatedly been identified as a source for resistance genes to pathogens (D'Costa et al., 2006, 2011; Finley et al., 2013; Martinez, 2008; Pruden et al., 2013; Wright, 2010), however, it is unclear to what extent antibiotics in the environment contribute to this development. Furthermore, current regulatory systems on pharmaceutical pollution do not account for resistance (Ashbolt et al., 2013; Boxall et al., 2012). In some cases, environmental concentrations close to, or exceeding, the minimal inhibitory concentrations (MICs) of certain antibiotics have been measured, generally linked to pollution from pharmaceutical production facilities (Larsson, 2014a), and often with drastic consequences in terms of resistance gene enrichments (Bengtsson-Palme et al., 2014b; Khan et al., 2013; Kristiansson et al., 2011; Liu et al., 2012; Wang et al., 2015). It is, however, well-known that antibiotic concentrations below the MICs can select for resistant bacteria (Andersson and Hughes, 2012; Gullberg et al., 2011; Gullberg et al., 2014; Liu et al., 2011). Although laboratory experiments have provided important insights into resistance evolution and revealed a previously unexplored landscape of sub-lethal resistance selection, their use for implementation of mitigation strategies for environmental releases of antibiotics is not straightforward. The reliability of the minimal selective concentrations (MSCs) obtained from competition experiments between two closely related strains is likely to be limited when extended to more complex microbial communities, as stronger selective forces, such as nutrient availability and predation, are likely to dominate at low antibiotic concentrations, as observed for many of other toxicants (Bengtsson-Palme et al., 2014a). In addition, the parallel competition between many species and genotypes makes it difficult to assess to what extent resistant genotypes will fill the niches made available by antibiotic selection. At the same time, a complex community

Abbreviations: EC50, 50% effect concentration; GMP, Good manufacturing practice; LOEC, Lowest effect concentration; MIC, Minimal inhibitory concentration; MSC, Minimal selective concentration; NOEC, No effect concentration; PNEC, Predicted no effect concentration; STP, Sewage treatment plant.

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may contain species and genotypes that are considerably more sensitive than those investigated in laboratory-based competition experiments with individual strains, creating opportunities for more tolerant bacteria to take their place (O'Brien, 2002; Zhang et al., 2011). To experimentally determine the MSCs in complex microbial systems is, however, labor-intensive, and the MSCs obtained would be expected to vary depending on the investigated test system. Nonetheless, attempts at determining the MSCs of specific antibiotics in complex systems have been made (Quinlan et al., 2011), but there is an urgent need for establishment of predicted no-effect concentrations (PNECs) and emission limits based on scientific data, and the consequences involved in not regulating releases of antibiotics into the environment could further escalate a problem that already has reached very serious proportions (Bengtsson-Palme and Larsson, 2015). In the light of this, attempts to theoretically determine the MSCs of various antibiotics have been suggested (Ågerstrand et al., 2015). Such approaches have previously been employed for a limited set of antibiotics, revealing that certain environments may harbor concentrations of antibiotics high enough to exert a selective pressure on clinically relevant bacteria (Tello et al., 2012). In this work, we have therefore broadly estimated MSCs using the EUCAST database (European Committee on Antimicrobial Susceptibility Testing, 2014), containing data on the minimal inhibitory concentrations of a range of clinically relevant bacteria. By taking advantage of the fact that an antibiotic concentration that kills or inhibits growth of at least some bacteria will, by consequence, be selective at the community level, we have determined the upper boundaries for MSCs, and suggested individual safety margins for antibiotics based on the extent of available MIC data. The resulting data can be used as guidance in environmental risk assessment, for regulatory bodies implementing emission limits of antibiotics into the external environment, as input to proposed environmental certificates within the good manufacturing practice (GMP) framework, and serve as a comprehensive reference framework for future studies on environmental antibiotic resistance.

2. Material and methods

2.1. Minimal inhibitory concentration data

Data on minimal inhibitory concentrations were obtained from the EUCAST database on 2014–11-26, containing minimal inhibitory concentration (MIC) data for 122 antibiotics/antibiotics combinations (Table S1) and 170 species (Table S2). Note that for each antibiotic, MIC data was only available for a subset of these 170 species. For each antibiotic, the lowest minimal inhibitory concentration was determined by: 1) removing all MIC values above the wildtype/resistance cutoff (ECOFF), to exclude data from resistant isolates; 2) finding the lowest MIC value for which there were ten or more observations at this concentration or lower, to reduce the risk of including individual, low values reported from determinations that might have been flawed despite the standard protocols followed to generate data; and 3) reporting the $MIC_{1\%}$, $MIC_{5\%}$, $MIC_{10\%}$ or $MIC_{50\%}$ values, corresponding to the value containing the bottom 1, 5, 10 or 50% of the MIC values, respectively, while satisfying criteria 1 and 2. The $MIC_{1\%}$ value for each antibiotic will be referred to as the "observed lowest MIC" throughout the paper. Finally, for combinations of species and antibiotics where the lowest MIC value was 2 μg/L, corresponding to the lowest reported concentrations in EUCAST, the lowest MIC was predicted by calculating the average log2-distance between the peak MIC value of the sensitivity distribution and the lowest MIC value for that antibiotic across all other species. Thereafter, the lowest MIC was extrapolated to be at the same log2-distance below the peak MIC. In cases where this predicted lowest MIC was higher than 2 μg/L, 2 μg/L was instead used as the "predicted" lowest MIC.

2.2. Taxonomic inference

To evaluate the influence of taxonomic dissimilarity between two species on the difference in lowest MIC values between the same species pair, the average SSU rRNA pairwise dissimilarity and the difference in lowest MIC values were compared for each antibiotic and each species. Species names from the EUCAST database were manually matched to the species names in the SILVA database (Yilmaz et al., 2014). All SSU rRNA sequences for each EUCAST species that could be matched to a species name in SILVA (85.6%; Table S3) were extracted from the SILVA SSU release 119 Ref (NR), as of 2014–12-01, resulting in 12,762 sequences (Item S1). Sequences that were indicated as having bad quality (SILVA sequence quality, alignment quality or pintail quality scores below 75), as well as sequences shorter than 1200 bp, were removed, resulting in 11,183 sequences that were downloaded for further analysis (Item S2). Species that did not have any sequence included after quality filtering (*Clavispora lusitaniae* and *Moraxella catarrhalis*; both excluded due to low pintail quality) had their sequences re-included in the dataset, resulting in 11,198 sequences in total (Item S3). Those sequences were run through Metaxa2 (Bengtsson-Palme et al., 2015b) version 2.0.2 (additional options "–cpu 16 –align none") to confirm their species identity, make sure all sequences were oriented in the forward direction, and to extract the SSU genes without their flanking regions from the sequences in cases where these were present in the SILVA database. The extracted SSU regions were clustered into 99% identity clusters using Usearch version 7.0.1090 (Edgar, 2010) to discard sequences differing mainly due to length variations and sequencing errors (options "-cluster_fast input_file -id 0.99 -centroids output_file"). The resulting sequences were aligned using MAFFT version 7.130b (additional options "–reorder –auto") and the pairwise sequence dissimilarities were determined, measured as the number of non-identical base pairs (including gaps) per total length.

2.3. Relating MIC difference to taxonomic dissimilarity

The influence of taxonomic divergence on the MSC upper boundaries was assessed using Pearson correlation between rRNA dissimilarity and difference in lowest MIC, calculated separately for each antibiotic. In addition, linear models were fitted to these data using iteratively reweighted least squares, to evaluate if rRNA dissimilarity could predict lowest MIC differences between species. Each regression model was tested for heteroscedasticity using the Breusch-Pagan (Cook-Weisberg) test as implemented in the R package car (Fox and Weisberg, 2011) to further identify effects of rRNA dissimilarity on lowest MIC distributions. The p-values for non-zero linear relationships and heteroscedasticity were corrected for multiple testing using the Benjamini-Hochberg false discovery rate with a significance cutoff of 0.05 (Benjamini and Hochberg, 1995). Finally, the taxonomic sampling coverage was estimated at the phylum, class, order and family levels for each antibiotic in the EUCAST database, to discern the degree of taxonomic bias for the MIC distributions of different antibiotics.

2.4. Accounting for small MIC sample sizes

To evaluate the uncertainty of the MSC upper boundaries, each antibiotic with more than 30 tested species was subjected to a resampling analysis, in which subsamples ranging from one to 30 lowest MIC values for different species were selected using the gdata R package (Warnes et al., 2013), noting the lowest MIC obtained for each subset. The obtained resampled lowest MICs for subsamples were then used to calculate size-adjusted lowest MICs for each antibiotic with less than 40 tested species, using the following formula:

$$[\text{observed/predicted lowest MIC}] \times [\text{number of tested species}]/41$$

where 41 is a constant determined from the resampling data. This resulted in size-adjusted lowest MICs, i.e. a prediction of the concentration at which 99% of bacterial isolates would have a higher MIC (since this number was derived from the $MIC_{1\%}$ above). Finally, to arrive at a PNEC, we applied a flat assessment factor of 10 to each size-adjusted lowest MIC, to account for the difference between inhibitory concentration and selective concentration of antibiotics (Andersson and Hughes, 2012: see also the Discussion section). The obtained values for size-adjusted predicted lowest MICs (upper MSC boundaries) and PNECs were then rounded down to the closest concentration on the EUCAST testing scale (in essence a two-fold dilution series corresponding to a log2-scale). We finally compared our obtained MSC boundaries and PNECs to the NOEC/EC50 and PNEC values reported in FASS (http://fass.se; 2015–08-26), and to the highest reported concentrations of antibiotics in effluents from conventional sewage treatment plants, as collected by Michael et al. (2013).

All analyses were carried out using Perl and R (R Development Core Team, 2011). The Perl and R scripts used to analyze the data are available as a supplementary item (Item S4).

3. Results

3.1. Taxonomic coverage of the EUCAST database

Although the 170 species present in the EUCAST database cover a wide range of microbial taxonomic groups, the vast majority of those are of clinical origin, and are to some degree pathogenic (Table S2). There is also apparent bias in terms of which antibiotics that have been tested and how many isolates that have undergone MIC testing (Fig. S1). Particularly overrepresented are the Bacilli, encompassing e.g. Staphylococcaceae and Streptococcaceae, and the Gammaproteobacteria, containing the Enterobacteriaceae family. This sampling bias is most evident for the number of tested isolates; the different types of antibiotics tested and reported in the EUCAST database are more evenly distributed across taxa. Importantly, although some antibiotics only have been tested against species from one or two families, the majority has been tested against at least five different families (Fig. S2). Particularly, the most commonly used antibiotics in clinical settings also had among the widest distributions of tested families (Fig. S3).

3.2. Observed lowest minimal selective concentrations

Lowest MIC values were obtained for 111 antibiotics and 11 antibiotics combinations in the EUCAST database (Table 1). For 13 antibiotics, the lowest MIC corresponded to the lowest concentration tested (2 μg/L) and for those, predicted lowest MICs were estimated by extrapolating the log2-distance below the peak MIC value and the lowest MIC value for that antibiotic across all species (Table 1). This resulted in six antibiotics having predicted lowest MICs slightly below 2 μg/L. Overall, the lowest MICs ranged from 0.69 μg/L (predicted concentration for ceftriaxone in *Neisseria meningitidis*) to 32,000 μg/L (clavulanic acid in *Acinetobacter baumannii*; Fig. 1 and Table 1). Most lowest MICs were in the range between 4 and 125 μg/L. The values reported here as the observed lowest MICs correspond to that 1% of isolate observations were at or below the reported concentration. However, the distributions of lowest MIC values were relatively stable, regardless of whether a 1%, 5% or 10% cutoff was used (Fig. S4; Table S4).

3.3. Inhibitory concentrations are only weakly linked to taxonomic divergence

Taxonomic distance between two species may be linked to the difference in lowest MICs observed. Thus, the relationship between rRNA dissimilarity (as a proxy for taxonomic divergence) and the log2 difference in lowest MIC was investigated for each antibiotic, and for all antibiotics together (Fig. 2). The overall link between rRNA dissimilarity and lowest MIC difference was very weak ($R^2 = 0.02$). Nonetheless, eleven individual antibiotics had significant relationships between rRNA dissimilarity and lowest MIC (Table S5). However, out of those eleven, five counter-intuitively had negative slopes (cefepime, ciprofloxacin, clindamycin, gentamicin, and norfloxacin), suggesting that more divergent species would have more similar lowest MICs than closely related ones. For the remaining six antibiotics, the degree to which lowest MIC differences could be explained by rRNA dissimilarity was very different, with R^2 values ranging from 0.23 to 0.97 (Table S5; Fig. S5).

Another possible consequence of a relationship between taxonomic divergence and lowest MIC difference is that the difference in lowest MIC would show more variation the larger the taxonomic distance between species. This would show in the data as increasing scattering (heteroscedasticity) when lowest MIC difference is plotted against rRNA dissimilarity. We did, however, not find significant heteroscedasticity for any antibiotic after correction for multiple testing (Table S5). Taken together, this suggests that the link between taxonomic distance and lowest MIC is weak, although it may exist to a minor degree for some antibiotics.

3.4. Predicted lowest minimal inhibitory concentrations

Since the number of species that each antibiotic had been tested against differed substantially, we used subsamples of the lowest MIC data for the antibiotics that had been tested against more than 30 species to assess the effect of small sample size on the estimated MSC boundaries (Fig. 3). The subsampling revealed that we consistently overestimated the lowest MIC for small samples sizes, but that this effect was minor for samples of size 20 and larger. We used this result to calculate how much lower the actual lowest MIC could be for antibiotics with small number of tested species, in case the EUCAST data happen to correspond to the upper part of the sensitivity distribution (Table 1). Still, if an antibiotic has been tested against a limited diversity of microorganisms, this may bias the lowest MIC estimate and PNECs, and we have therefore also provided the number of genera and families that each antibiotic has been tested against in Table 1.

3.5. Predicted no effect concentrations (PNECs) for resistance

To predict no effect concentrations for resistance selection for each antibiotic, we used the sample size adjusted lowest MICs and applied an assessment factor of 10 to account for that the selective concentration must be lower than the MIC. The PNECs ranged from 0.008 μg/L to 64 μg/L (Fig. 4), as compared to the observed/predicted lowest MICs, ranging from 0.69 μg/L to 32,000 μg/L. Generally, the PNECs obtained after sample size adjustment and application of the assessment factor were about 16–32 times smaller than the observed lowest MICs, although a large number of antibiotics had a 500-fold difference, as a result of few empirical MIC values. Accordingly, the antibiotics that had been tested against the largest number of species generally had 16-fold differences between PNEC and lowest MIC. Comparing these estimated lowest MICs and PNECs to available data on lowest effect concentrations (LOECs) or PNECs was in most cases not possible, since LOEC data was present in FASS only for 21% and PNEC data for 17% of the investigated antibiotics. Only in two cases (moxifloxacin and sulfamethoxazole), the size-adjusted lowest MICs were higher than the LOECs currently presented in FASS based on ecotoxicological testing. In five cases, the PNECs we report exceeded the corresponding PNECs in FASS (clarithromycin, erythromycin, roxithromycin, sulfamethoxazole, and telithromycin), while in 15 cases the PNEC reported in FASS were higher (102 antibiotics lacked PNEC data in FASS).

We finally compared our PNECs to the concentrations of antibiotics that have been measured in effluents from conventional sewage treatment plants (Table 1). For the 32 antibiotics where such

Table 1
Estimated minimal selection concentration boundaries (in μg/L) and predicted no-effect concentrations for 111 antibiotics and 11 antibiotics combinations.

Antibiotic	N[1]	Observed lowest MIC[2]	Predicted lowest MIC[3]	Size-adjusted lowest MIC[4]	PNEC (resistance selection)[5]	Covered genera (families)[6]	NOEC (ecotox)[7]	PNEC (ecotox)[8]	STP effluent conc.[9]
Amikacin	28	250		125	16	15 (8)			
Amoxicillin	29	4		2	0.25	19 (12)			0.05
Amoxicillin–clavulanic acid (fixed)	4	1000		64	8	4 (2)			
Amphotericin B	1	8		0.125	0.016	1 (1)			
Ampicillin	64	4		4	0.25	25 (15)	>1,000,000		0.126
Ampicillin–sulbactam (fixed)	3	500		32	2	3 (1)			
Ampicillin–sulbactam (ratio)	23	125		64	4	13 (6)			
Anidulafungin	4	2	2	0.125	0.016	1 (1)			
Avilamycin	6	1000		125	8	2 (2)			
Azithromycin	12	16		4	0.25	6 (6)			0.38
Aztreonam	11	32		8	0.5	10 (5)			
Bacitracin	2	2000		64	8	1 (1)			
Benzylpenicillin	47	4		4	0.25	12 (11)			
Capreomycin	1	1000		16	2	1 (1)			
Cefaclor	11	32		8	0.5	7 (6)			1.8
Cefadroxil	7	125		16	2	5 (4)			
Cefalexin	10	250		32	4	7 (5)			1.8
Cefaloridine	1	2000		32	4	1 (1)			
Cefalothin	13	64		16	2	10 (4)			
Cefazolin	18	32		8	1	12 (6)			
Cefdinir	5	32		2	0.25	4 (4)			
Cefepime	41	8		8	0.5	18 (10)			
Cefepime–clavulanate	1	1000		16	2	1 (1)			
Cefixime	11	4		1	0.064	7 (6)			
Cefoperazone	13	16		4	0.5	10 (5)			
Cefotaxime	33	2	1.8	1	0.125	19 (10)	>500,000[a]		0.034
Cefotaxime–clavulanate	2	8		0.25	0.032	2 (2)			
Cefoxitin	26	250		125	8	13 (5)			
Cefpirome	9	4		0.5	0.064	7 (5)			
Cefpodoxime	15	8		2	0.25	10 (7)			
Cefpodoxime–clavulanic acid	3	250		16	1	2 (1)			
Ceftaroline	8	4		0.5	0.064	5 (4)			
Ceftazidime	23	16		8	0.5	17 (9)	13	1.3	
Ceftazidime–clavulanate	1	8		0.125	0.016	1 (1)	13	1.3	
Ceftibuten	16	8		2	0.25	13 (6)	600,000[b]		
Ceftiofur	4	8		0.5	0.064	3 (3)			
Ceftobiprole	39	4		2	0.25	17 (9)			
Ceftriaxone	29	2	0.69	0.25	0.032	16 (10)			
Cefuroxime	29	8		4	0.5	16 (8)			
Chloramphenicol	29	125		64	8	18 (11)			
Ciprofloxacin	70	2	1.2	1	0.064	29 (18)	1.2	1.2	0.742
Clarithromycin	15	8		2	0.25	10 (10)	2[b]	0.04	0.61
Clavulanic acid	1	32,000		500	64	1 (1)			
Clinafloxacin	7	32		4	0.5	4 (4)			
Clindamycin	37	16		8	1	12 (11)			0.07
Cloxacillin	1	64		1	0.125	1 (1)			0.7
Colistin	16	64		16	2	10 (4)			
Daptomycin	16	32		8	1	6 (6)			
Doripenem	39	2	2	1	0.125	18 (10)			
Doxycycline	29	32		16	2	20 (11)			0.915
Enrofloxacin	4	8		0.5	0.064	4 (3)			0.05
Ertapenem	36	2	2	1	0.125	20 (12)	100,000	500	
Erythromycin	39	16		8	1	14 (13)	10.3	0.103	0.62
Ethambutol	1	1000		16	2	1 (1)			
Faropenem	1	8		0.125	0.016	1 (1)			
Fidaxomicin	1	8		0.125	0.016	1 (1)			
Florfenicol	9	125		16	2	6 (5)			
Fluconazole	3	64		4	0.25	1 (1)			
Flumequine	3	64		4	0.25	3 (2)			
Fosfomycin	13	125		32	2	9 (6)			
Fusidic acid	9	32		4	0.5	4 (4)	4300	4.3	
Gatifloxacin	21	4		2	0.125	14 (10)			0.056
Gemifloxacin	16	2	2	0.5	0.064	11 (10)			
Gentamicin	68	16		16	1	27 (14)			1.3
Imipenem	53	2	2	2	0.125	23 (14)	>78,000[a]	78	
Isoniazid	1	64		1	0.125	1 (1)	406[b]		
Itraconazole	1	4		0.064	0.008	1 (1)	1,000,000	1000	
Kanamycin	13	125		32	2	10 (7)			
Ketoconazole	*	4		*	*	0 (0)			
Levofloxacin	43	4		4	0.25	24 (16)	7400[b]	7.4	
Lincomycin	2	500		16	2	2 (2)			0.3
Linezolid	29	125		64	8	9 (9)			
Loracarbef	10	125		16	2	8 (4)			

Table 1 (*continued*)

Antibiotic	N[1]	Observed lowest MIC[2]	Predicted lowest MIC[3]	Size-adjusted lowest MIC[4]	PNEC (resistance selection)[5]	Covered genera (families)[6]	NOEC (ecotox)[7]	PNEC (ecotox)[8]	STP effluent conc.[9]
Mecillinam	9	64		8	1	6 (2)			
Meropenem	50	2	0.88	0.5	0.064	22 (14)	3.6	1.5	
Metronidazole	6	16		2	0.125	3 (3)	2030[c]	40.6	0.561
Micafungin	*	4		*	*	0 (0)			
Minocycline	24	32		16	1	15 (8)			<0.03
Moxifloxacin	53	2	2	2	0.125	21 (14)	1.8	0.18	0.017
Mupirocin	4	32		2	0.25	2 (2)	1,000,000		
Nalidixic acid	17	500		125	16	13 (5)			0.45
Narasin	2	125		4	0.5	1 (1)			
Neomycin	8	125		16	2	6 (5)			
Netilmicin	14	16		4	0.5	9 (8)			
Nitrofurantoin	8	4000		500	64	5 (4)			
Norfloxacin	15	16		4	0.5	12 (8)			0.32
Ofloxacin	26	8		4	0.5	20 (14)			4.82
Oxacillin	24	32		16	1	5 (5)			0.008
Oxytetracycline	2	125		4	0.5	2 (2)			0.07
Pefloxacin	1	4000		64	8	1 (1)			
Phenoxymethylpenicillin	8	4		0.5	0.064	5 (5)			2
Piperacillin	30	8		4	0.5	18 (11)			
Piperacillin–tazobactam	43	4		4	0.25	20 (13)			
Quinupristin–dalfopristin	17	64		16	2	4 (4)			
Retapamulin	4	8		0.5	0.064	2 (2)	100		
Rifampicin	19	2	1.7	0.5	0.064	12 (12)	3,300,000[b]	3300	
Roxithromycin	14	32		8	1	7 (7)	10	0.047	0.54
Secnidazole	1	500		8	1	1 (1)			
Sparfloxacin	23	2	1.8	1	0.064	17 (13)			
Spectinomycin	8	2000		250	32	5 (4)			
Spiramycin	2	125		4	0.5	1 (1)			0.454
Streptomycin	32	250		125	16	14 (9)			
Sulbactam	12	1000		250	16	10 (5)			
Sulfamethoxazole	8	1000		125	16	6 (4)	5.9	0.59	0.964
Teicoplanin	19	16		4	0.5	4 (4)			
Telithromycin	11	4		1	0.064	8 (8)	2.38	0.0024	
Tetracycline	66	16		16	1	30 (18)			0.62
Thiamphenicol	1	500		8	1	1 (1)			
Tiamulin	1	500		8	1	1 (1)			
Ticarcillin	16	250		64	8	9 (5)			
Ticarcillin–clavulanic acid	14	64		16	2	9 (5)			
Tigecycline	54	16		16	1	26 (16)			
Tilmicosin	2	250		8	1	2 (1)			
Tobramycin	31	16		8	1	15 (8)	51	5.1	
Trimethoprim	22	16		8	0.5	15 (7)	5600	56	2.4
Trimethoprim–sulfamethoxazole	36	8		4	0.5	22 (13)	5.9	0.59	
Trovafloxacin	3	8		0.5	0.032	3 (3)			
Tylosin	1	2000		32	4	1 (1)			3.4
Vancomycin	42	125		125	8	10 (9)			0.04
Viomycin	1	1000		16	2	1 (1)			
Virginiamycin	5	250		16	2	3 (3)			
Voriconazole	*	2	2	*	*	0 (0)			

Notes: All concentrations are given in μg/L.

[1] These numbers correspond to the number of different species present in EUCAST that could be matched to a valid species name in SILVA. Thus, this number is sometimes zero (indicated by *), and in those cases we only report the observed/predicted lowest MIC (corresponding to the MSC upper boundary).

[2] The lowest MIC value observed for any species in the EUCAST database.

[3] Predicted lowest MIC values in cases where the lowest MIC in the EUCAST database equaled the lowest concentration tested (2 μg/L).

[4] The size-adjusted lowest MIC prediction, corresponding to the estimated upper boundary for the MSC (rounded down to the closest concentration on the EUCAST testing scale).

[5] The PNEC corresponds to the size-adjusted lowest MIC divided by an assessment factor of 10 (rounded down to the closest concentration on the EUCAST testing scale).

[6] The number of different genera and families tested against the antibiotic in the EUCAST database.

[7] NOEC in FASS derived from ecotoxicological data. The NOEC column also represents LC50, EC50 and EC10 data when NOEC data was not available (see notes).

[8] Current PNEC present in FASS, based on ecotoxicological data.

[9] The highest concentration observed in effluents from conventional STPs as reported by Michael et al. (2013).

[a] LC50.

[b] EC50.

[c] EC10.

measurement data was available, we found that in nine cases the highest measured effluent concentration exceeded the PNEC for that antibiotic, indicating that in some occasions effluent from sewage treatment may have the potential to be selecting for antibiotic resistance. In the majority of these cases, there were no LOEC or PNEC data available in FASS. Additionally, in the cases of ciprofloxacin, ofloxacin and phenoxymethylpenicillin the measured concentrations were close to the observed MICs for these antibiotics, suggesting that effluent concentrations of these antibiotics could be high enough to inhibit growth of or kill bacteria.

4. Discussion

In this work we provide an extensive number of theoretically determined selective concentrations, based on observed lowest MICs from the EUCAST database. These estimated upper boundary MSCs

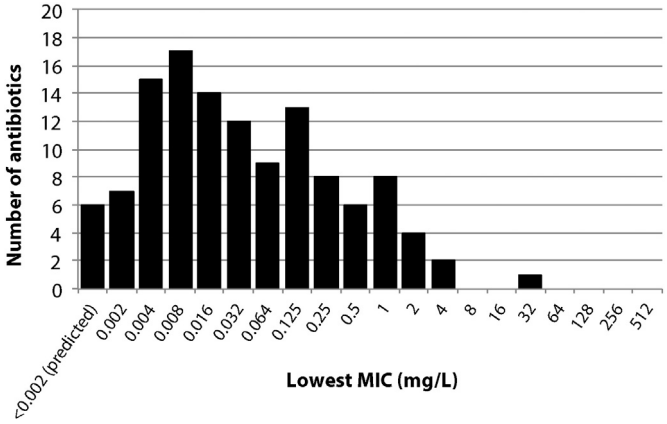

Fig. 1. Distribution of predicted and observed lowest MICs for all antibiotics.

can be seen as analogous to LOEC (Lowest Observed Effect Concentration) values used in environmental risk assessment for different chemicals. We have accordingly estimated PNECs (Predicted No Effect Concentrations) for resistance selection in microbial communities to be applied in regulatory contexts. These PNECs can eventually be refined or supplemented with data on experimentally derived selective concentrations in microbial communities as such data become available. Specifically, the PNECs can be used for antibiotic-producing companies to assess and manage risks for resistance selection associated with their own discharges (Murray-Smith et al., 2012), or for local authorities to define emissions limits of such factories. The PNECs also fill an important knowledge gap in order to make proposed environmental certificates within the good manufacturing practice framework for antibiotics concrete (Larsson, 2014a; Swedish Medical Products Agency, 2009, 2011; Pruden et al., 2013). Similarly, further development of the environmental criteria during public procurement processes for antibiotics, as already implemented by Sweden (Laurell et al., 2014) and considered by the WHO (SPHS Secreteriat, UNDP Istanbul Regional Hub, 2015), will eventually require defined discharge limits. With regards to environmental monitoring programs, PNECs can provide input to acceptable detection limits, and comparisons between PNECs and predicted or measured environmental concentrations can identify antibiotics of particular concern. The latter comparison can also provide input to possible regulations of antibiotics in surface waters, as currently considered within the water framework directive (Carvalho et al., 2015) and to improve the environmental risk assessment of pharmaceuticals (Ågerstrand et al., 2015).

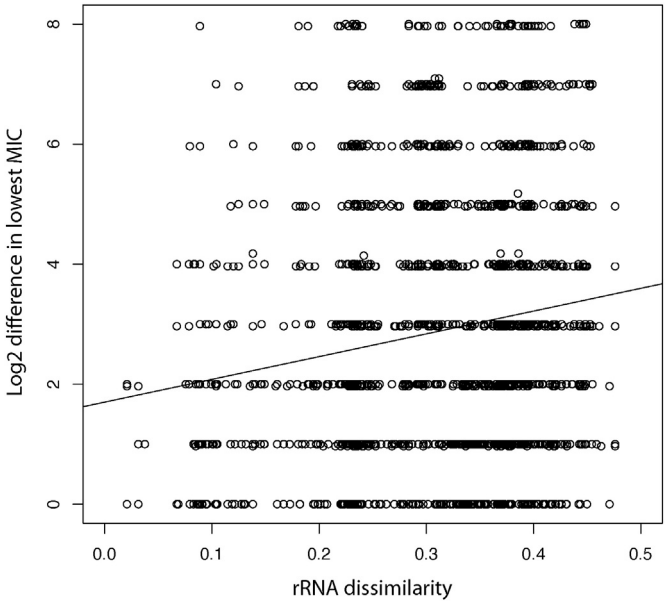

Fig. 2. Linear regression between rRNA dissimilarity and difference in lowest MIC for all antibiotics together.

4.1. Estimating MSCs and PNECs

The upper boundaries for MSCs we report in this work are based on the simple assumption that an antibiotic concentration that inhibits growth of some bacteria will by consequence have selective effects on the community level, at least in some bacterial communities (Ågerstrand et al., 2015). The estimation of MSC data from the MICs reported in EUCAST is influenced by at least three major factors; a possible connection between taxonomic divergence and antibiotic susceptibility, limited taxonomic sampling coverage for many antibiotics in EUCAST, and the fact that antibiotics have selective effects at sub-inhibitory concentrations (Chow et al., 2015; Gullberg et al., 2011, 2014).

The PNECs of this study are based on the assumption that the species present in the EUCAST database are to some extent representative of the diversity of sensitive bacteria in nature. It is therefore important to quantify the relationship between taxonomic divergence and the degree of similarity in terms of antibiotic susceptibility. We found that the degree to which susceptibility can be explained by taxonomic similarity of the species covered in the database is limited. Such links may still exist for certain antibiotics, but might be obscured since completely insensitive species are not included in the database. The absence of a strong relationship suggests that our results may hold for a majority of the antibiotics evaluated, and we have therefore chosen not to include any assessment factor for the taxonomic span for which different antibiotics were tested against. It should be noted that many antibiotics have narrow spectra and cannot be expected to have been tested against a broad range of microbial taxa, as such testing would in many cases be clinically irrelevant. For our approach, including such presumably insensitive species would not lead to lower minimal MICs.

Many antibiotics have been tested against a very limited number of species, and there is indeed an inverse correlation between the *number* of tested species and the observed lowest MIC, suggesting that smaller number of tested species would result in a bias toward greater MSCs and PNECs. As a remedy, we used data from resampling of the antibiotics tested against more than 30 species to determine to which degree we over-estimated the lowest MIC at different sample sizes. In this way, we arrived at size-adjusted predicted lowest MICs (upper boundaries for MSCs), accounting for limited diversity in terms of tested species. Nevertheless, we expect the PNEC estimate to be more accurate the more genera and families an antibiotic has been evaluated against.

Finally, antibiotics tend to have selective ability at concentrations below their MICs, and since our PNECs are derived from MIC data, this factor also needs to be taken into account. Risk management always involves dealing with uncertainties while trying to strike a balance between the probability for an event to occur, the severity of the potential outcome and the costs or consequences involved in managing the risk (Chapman et al., 1998). The size of an assessment factor is therefore context dependent, and will be a reflection of how far one wants to enforce the precautionary principle. Gullberg et al. (2011) report the MSCs to be in the range of 1/230 to 1/4 of the MIC for different antibiotics in experiments with two competing bacterial strains. Based on these findings, an assessment factor of up to 230 could be warranted. However, establishing a PNEC by selecting the lowest MIC of the most sensitive species in EUCAST, adjusting that concentration for limited sampling size, and then assuming the maximal described difference between the size-adjusted MIC and MSC could be overly conservative. Instead, we

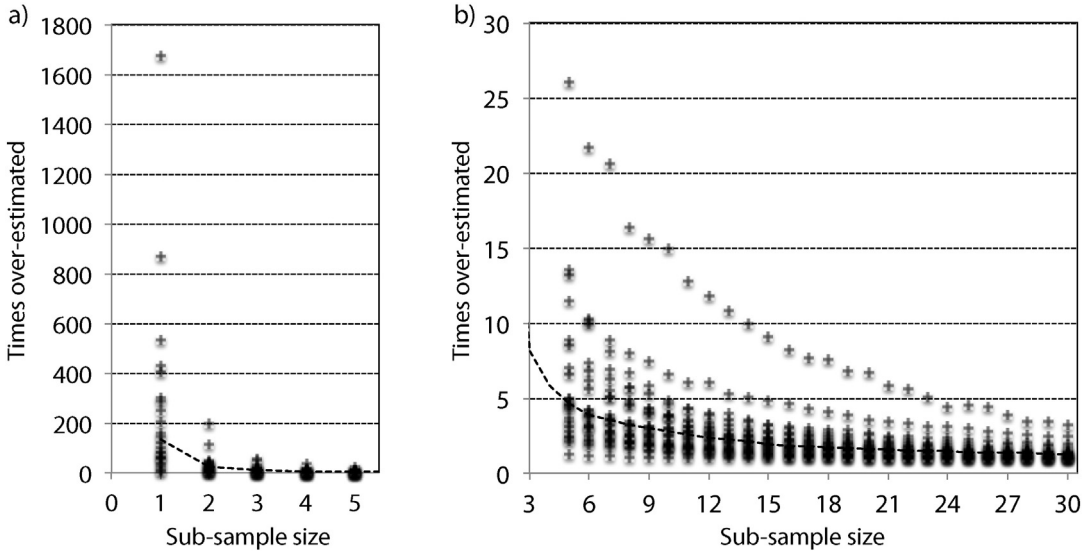

Fig. 3. Degree of lowest MIC over-estimation for subsampled datasets. (a) Subsamples of size one to five, (b) subsamples of size five to thirty. The dashed line represents the median of observations.

propose a more modest assessment factor of 10 – corresponding approximately to the median MIC/MSC ratio reported by Gullberg et al. (2011). We acknowledge that the relationship between the MIC and the MSC is the most uncertain factor in the estimation process and that we may very well underestimate this difference for some antibiotics. Depending on the context, additional safety margins could be employed, for example if there are reasons to believe that certain environments need particular protection, or if there is experimental data suggesting high selective potency for certain antibiotics. Given the moderate assessment factor proposed, we do not think limits on environmental exposure should exceed the PNECs unless there are strong and relevant experimental evidence for lack of effects at higher concentrations.

4.2. Ecological relevance of the PNECs

Environmental concentrations of antibiotics have been shown to vary substantially. For example, ciprofloxacin has been detected at a concentration of 0.026 μg/L in surface water in Italy (Calamari et al., 2003), 0.009 μg/L in Germany (Christian et al., 2003), 0.11 μg/L in Chinese river water (Luo et al., 2011), and up to 2.5 μg/L in Indian well water close to pharmaceutical industries (Fick et al., 2009). Our PNEC for ciprofloxacin is 0.064 μg/L, meaning that in the Chinese and Indian cases, surface water concentrations may be selective over extended time periods. The predicted no-effect concentration for ciprofloxacin that can be derived from Tello et al. (2012) is around 0.1 μg/L, which is only somewhat higher than our PNEC, but ten times lower than our estimate for the upper MSC boundary. This can be compared to the concentrations measured in effluent from pharmaceutical production (31,000 μg/L (Larsson et al., 2007)), the receiving river (2500 μg/L (Fick et al., 2009)), and in lakes subjected to dumping of pharmaceutical waste (6500 μg/L (Fick et al., 2009)). In all these cases, the measured concentrations are readily above the estimated MSC upper boundary, and many times well above the MICs for ciprofloxacin for most investigated species.

Municipal sewage treatment plants (STPs) are considered to be important point sources of antibiotic releases into the environment (Michael et al., 2013). When we compared chemical data from STP effluents to our PNECs, we discovered that in 28% of cases the highest reported effluent concentration exceeded the PNEC. However, measured concentrations were generally well below the MSC upper boundary estimates, even in the worst cases. This suggests that conventional STPs could, in some cases, facilitate selection for antibiotic resistance genes, and indicates that there is a need to evaluate advanced treatment strategies to avoid resistance gene enrichment. It should be noted, though, that the dilution of the effluent in the recipient will often be large, and hence disinfection of effluents, rather than removal of selective agents, might be a more important measure. The PNEC data provided in this study could aid in decisions on international, national and local levels on which extended treatment processes that would be desirable to implement, and if there is a need for disinfection, or other sanitary interventions, of STP effluents.

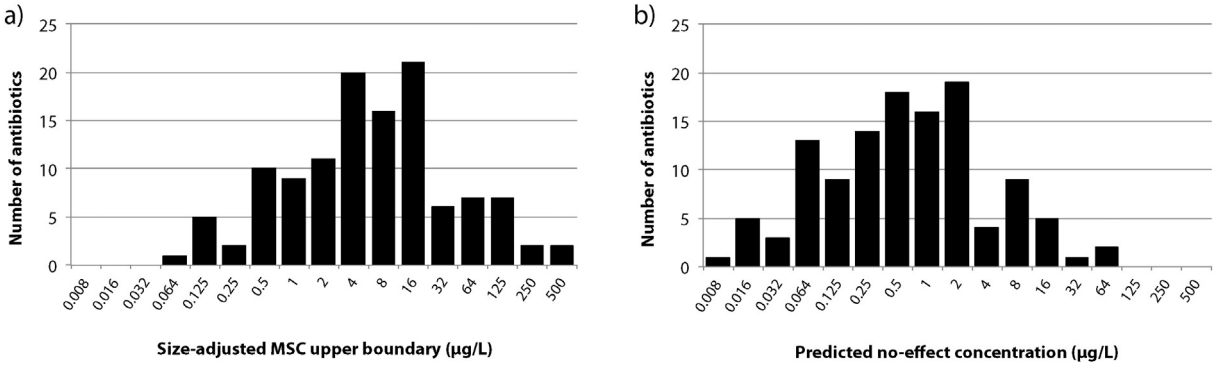

Fig. 4. Distribution of size-adjusted lowest MICs (MSC upper boundaries) (a) and predicted no-effect concentrations (b) for all antibiotics.

MIC tests measure acute effects on bacteria rather than long-term, and also measure growth inhibition under high nutrient availability. Thus, nearly all selection in the environment will occur during much longer timescales than under laboratory conditions, and the longer generation times may potentially narrow the sub-MIC selective window for many antibiotics. On the other hand, antibiotics such as tetracyclines and fluoroquinolones are not readily degraded in the environment and may persist for extended time periods, thus exerting a chronic selection pressure on microbial communities (Kümmerer, 2009), with unknown consequences for resistance development. Further complicating the issue of environmental selection is the limited knowledge of the influence of sorption of antibiotics to particles and whether adsorbed antibiotics may still exert an effect (Boxall et al., 2012; Chander et al., 2005; Córdova-Kreylos and Scow, 2007). In addition, bacteria in the environment are likely to be exposed to mixtures of antibiotics rather than single substances, which may further lower the MSCs (Gullberg et al., 2014). Although there is a growing body of research on combination effects of antimicrobial substances on bacteria (Backhaus, 2014; Brosché and Backhaus, 2010; Christensen et al., 2006), the understanding of such effects on selection for resistance in microbial communities is limited. Furthermore, biocides and metals may also contribute to the selection of antibiotic resistance genes (Pal et al., 2014). It is also possible that antibiotics may disturb ecosystem services such as sewage treatment, nitrogen fixation and nutrient fluxes (Larsson, 2014b), although there is little evidence that the levels of antibiotics present in the environment can have any significant effect on such processes. Indeed microbial communities seem to uphold a surprising degree of resilience even to high antibiotics exposure over long timeframes, possibly in part due to sharing of resistance factors through horizontal gene transfer (Bengtsson-Palme et al., 2014b; Flach et al., 2015; Jernberg et al., 2007; Lewis, 2007; Relman, 2012).

In addition to exerting a selection pressure for resistant strains, subinhibitory concentrations of antibiotics can have several other effects on bacterial communities (Rodríguez-Rojas et al., 2013). For example, effects on the mutation and recombination rates of bacteria have been observed for beta-lactams (Cortes et al., 2008; Gutierrez et al., 2013), ciprofloxacin (López et al., 2007; Morero et al., 2011), and many other antibiotics (Chow et al., 2015; Thi et al., 2011). Furthermore, subinhibitory concentrations of antibiotics have the potential to induce horizontal transfer of genetic material (Johnson et al., 2015; López and Blázquez, 2009; Prudhomme et al., 2006). These effects have partially been attributed to the bacterial SOS response (Beaber et al., 2004; Guerin et al., 2009). The SOS response can also lead to increased mutation rates through induction of error-prone DNA-polymerases, triggered by a range of antibiotics (Briales et al., 2012; Thi et al., 2011). The degree to which the SOS response is activated depends on antibiotic concentration (Dörr et al., 2009; Torres-Barceló et al., 2015), however, to our best knowledge studies establishing the minimal concentrations for its activation are lacking. For ciprofloxacin, concentrations demonstrated for induction (>1 μg/L) are several times above the PNEC we predict for resistance selection. Finally, antibiotics promote biofilm formation (Balaji et al., 2013; Hoffman et al., 2005), which may further enhance persistence during antibiotic selection. All these processes are to some degree involved in antibiotic resistance development, and it would thus be very valuable to establish the minimal concentrations of antibiotics that induce mutagenesis, transfer of genetic material between bacteria, mobilization of chromosomal DNA, and biofilm formation, as a complement to the determining concentrations that are directly selective for resistance.

4.3. Implications for regulation of antibiotic emissions

This work represents an approach to theoretically determine MSCs using observed MIC values, providing comprehensive data as a starting point for regulatory agencies. Our data suggests that emission limits for antibiotics must be set individually for each compound, and that different antibiotics have very different potential to be selective. Furthermore, some antibiotics, such as ciprofloxacin, can be detected in surface water at concentrations that our PNECs indicate would have potential to be selective. In this context it is important to recall that the transfer of a novel resistance determinant from an environmental bacteria to a human pathogen only need to occur once (Bengtsson-Palme and Larsson, 2015; Larsson, 2014b); given sufficient selection pressure dissemination and maintenance of this resistance factor may then be facilitated by human and veterinary drug use, insufficient hygiene standards, as well as global travel and trade (Ashbolt et al., 2013; Bengtsson-Palme et al., 2015a; Larsson, 2014a). Clearly, there are strong incentives to introduce evaluation based on available MSC data in the environmental risk assessment of antibiotics within guidelines of, for example, the European Medicine's Agency.

Importantly, current data available to decision makers to form a basis for regulatory efforts is very scarce. Few antibiotics have gone through ecotoxicological testing, and as seen in this study, even the PNECs reported in FASS are sometimes above the observed MICs for certain antibiotics, revealing an important discrepancy between ecotoxicological testing and clinical data on toxicity in specific pathogenic bacteria. Only in two cases (moxifloxacin and sulfamethoxazole) were our estimated MSC upper boundaries higher than the LOECs reported in FASS. In addition, only 21% of the antibiotics investigated in this work had any ecotoxicological data available. Establishment of MSCs for antibiotic resistance in environmental bacterial communities is therefore crucial to enable proper risk assessment to underpin regulatory interventions. In the absence of experimentally determined MSCs in complex microbial communities, the theoretical MSC boundaries and PNECs presented in this work can function as guidance. We would recommend regulators to consider the PNECs reported here as a basis for implementation of emission limits. Preferably, legislation should in the future be refined including experimental data directly assessing the MSCs as such data become available. Thus, experimental studies of establishment of complex microbial communities under different antibiotic exposure, followed by analyses of changes in taxa, resistance patterns or increasing mobility of genetic material, are desirable to further refine discharge standards. In the meantime, the PNECs we report here can be used to better focus mitigation strategies on environments where risks for resistance promotion are particularly high. Factors such as sanitation, hygiene, urbanization, sewage treatment, and pharmaceutical manufacturing have been identified to be important for the ecological footprints of pharmaceuticals in the environment (Kookana et al., 2014), and the same set of factors are likely to be involved in resistance development as well. However, to mitigate resistance dissemination, not only the discharges of antibiotics must be managed. Resistant pathogens may be released from sewage treatment processes, and resistant bacteria may also emerge from e.g. industrial processes. In these cases, management strategies need to involve both limits on antibiotic levels, based on concentrations that do not promote maintenance of resistant strains, as well as sanitary efforts to remove bacteria from effluents.

5. Conclusions

In this work, we have presented compound-specific estimates for minimal selective concentrations and predicted no-effect concentrations for antibiotics based on MIC data derived from the EUCAST database. The data presented can serve as guidance for efforts by industries, regulatory agencies or purchasers of medicines to define acceptable environmental emissions of antibiotics that take into account risks for resistance promotion. The data can also aid in the implementation of environmental monitoring programs, directing mitigations, and for prioritizing future studies on environmental antibiotic resistance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2015.10.015.

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References

Ågerstrand, M., Berg, C., Björlenius, B., Breitholtz, M., Brunstrom, B., Fick, J., Gunnarsson, L., Larsson, D.G.J., Sumpter, J.P., Tysklind, M., Rudén, C., 2015. Improving environmental risk assessment of human pharmaceuticals. Environ. Sci. Technol. http://dx.doi.org/10.1021/acs.est.5b00302.

Andersson, D.I., Hughes, D., 2012. Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resist. Updat. 15, 162–172. http://dx.doi.org/10.1016/j.drup.2012.03.005.

Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G.J., McEwen, S.A., Ryan, J.J., Schönfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E., 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ. Health Perspect. 121, 993–1001. http://dx.doi.org/10.1289/ehp.1206316.

Backhaus, T., 2014. Medicines, shaken and stirred: a critical review on the ecotoxicology of pharmaceutical mixtures. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369. http://dx.doi.org/10.1098/rstb.2013.0585.

Balaji, K., Thenmozhi, R., Pandian, S.K., 2013. Effect of subinhibitory concentrations of fluoroquinolones on biofilm production by clinical isolates of Streptococcus pyogenes. Indian J. Med. Res. 137, 963–971.

Beaber, J.W., Hochhut, B., Waldor, M.K., 2004. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427, 72–74. http://dx.doi.org/10.1038/nature02241.

Bengtsson-Palme, J., Larsson, D.G.J., 2015. Antibiotic resistance genes in the environment: prioritizing risks. Nat. Rev. Microbiol. 13, 396. http://dx.doi.org/10.1038/nrmicro3399-c1.

Bengtsson-Palme, J., Alm Rosenblad, M., Molin, M., Blomberg, A., 2014a. Metagenomics reveals that detoxification systems are underrepresented in marine bacterial communities. BMC Genomics 15, 749. http://dx.doi.org/10.1186/1471-2164-15-749.

Bengtsson-Palme, J., Boulund, F., Fick, J., Kristiansson, E., Larsson, D.G.J., 2014b. Shotgun metagenomics reveals a wide array of antibiotic resistance genes and mobile elements in a polluted lake in India. Front. Microbiol. 5, 648. http://dx.doi.org/10.3389/fmicb.2014.00648.

Bengtsson-Palme, J., Angelin, M., Huss, M., Kjellqvist, S., Kristiansson, E., Palmgren, H., Larsson, D.G.J., Johansson, A., 2015a. The human gut microbiome as a transporter of antibiotic resistance genes between continents. Antimicrob. Agents Chemother. 59, 6551–6560. http://dx.doi.org/10.1128/AAC.00933-15.

Bengtsson-Palme, J., Hartmann, M., Eriksson, K.M., Pal, C., Thorell, K., Larsson, D.G.J., Nilsson, R.H., 2015b. Metaxa2: improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data. Mol. Ecol. Resour. 15, 1403–1414. http://dx.doi.org/10.1111/1755-0998.12399.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Methodol. 57, 289–300.

Boxall, A.B.A., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., Ankley, G.T., Beazley, K.F., Belanger, S.E., Berninger, J.P., Carriquiriborde, P., Coors, A., Deleo, P.C., Dyer, S.D., Ericson, J.F., Gagné, F., Giesy, J.P., Gouin, T., Hallstrom, L., Karlsson, M.V., Larsson, D.G.J., Lazorchak, J.M., Mastrocco, F., McLaughlin, A., McMaster, M.E., Meyerhoff, R.D., Moore, R., Parrott, J.L., Snape, J.R., Murray-Smith, R., Servos, M.R., Sibley, P.K., Straub, J.O., Szabo, N.D., Topp, E., Tetreault, G.R., Trudeau, V.L., Van Der Kraak, G., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. http://dx.doi.org/10.1289/ehp.1104477.

Briales, A., Rodriguez-Martinez, J.M., Velasco, C., Machuca, J., Díaz de Alba, P., Blázquez, J., Pascual, A., 2012. Exposure to diverse antimicrobials induces the expression of qnrB1, qnrD and smaqnr genes by SOS-dependent regulation. J. Antimicrob. Chemother. 67, 2854–2859. http://dx.doi.org/10.1093/jac/dks326.

Brosché, S., Backhaus, T., 2010. Toxicity of five protein synthesis inhibiting antibiotics and their mixture to limnic bacterial communities. Aquat. Toxicol. 99, 457–465. http://dx.doi.org/10.1016/j.aquatox.2010.06.008.

Calamari, D., Zuccato, E., Castiglioni, S., Renzo Bagnati, A., Fanelli, R., 2003. Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy. Environ. Sci. Technol. 37, 1241–1248.

Carvalho, R.N., Ceriani, L., Ippolito, A., Lettieri, T., 2015. Development of the 1st Watch List Under the Environmental Quality Standards Directive (No. EUR 27142 EN). Publications Office of the European Union, Luxembourg http://dx.doi.org/10.2788/101376.

Chander, Y., Kumar, K., Goyal, S.M., Gupta, S.C., 2005. Antibacterial activity of soil-bound antibiotics. J. Environ. Qual. 34, 1952–1957. http://dx.doi.org/10.2134/jeq2005.0017.

Chapman, P.M., Fairbrother, A., Brown, D., 1998. A critical evaluation of safety (uncertainty) factors for ecological risk assessment. Environ. Toxicol. Chem. 17, 99–108. http://dx.doi.org/10.1002/etc.5620170112.

Chow, L., Waldron, L., Gillings, M.R., 2015. Potential impacts of aquatic pollutants: sub-clinical antibiotic concentrations induce genome changes and promote antibiotic resistance. Front. Microbiol. 6, 803. http://dx.doi.org/10.3389/fmicb.2015.00803.

Christensen, A.M., Ingerslev, F., Baun, A., 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. Environ. Toxicol. Chem. 25, 2208–2215.

Christian, T., Schneider, R.J., Färber, H.A., Skutlarek, D., Meyer, M.T., Goldbach, H.E., 2003. Determination of antibiotic residues in manure, soil, and surface waters. Acta Hydrochim. Hydrobiol. 31, 36–44. http://dx.doi.org/10.1002/aheh.200390014.

Córdova-Kreylos, A.L., Scow, K.M., 2007. Effects of ciprofloxacin on salt marsh sediment microbial communities. ISME J. 1, 585–595. http://dx.doi.org/10.1038/ismej.2007.71.

Cortes, P.R., Piñas, G.E., Albarracin Orio, A.G., Echenique, J.R., 2008. Subinhibitory concentrations of penicillin increase the mutation rate to optochin resistance in *Streptococcus pneumoniae*. J. Antimicrob. Chemother. 62, 973–977. http://dx.doi.org/10.1093/jac/dkn322.

D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D., 2006. Sampling the antibiotic resistome. Science 311, 374–377. http://dx.doi.org/10.1126/science.1120800.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. http://dx.doi.org/10.1038/nature10388.

Dörr, T., Lewis, K., Vulić, M., 2009. SOS response induces persistence to fluoroquinolones in *Escherichia coli*. PLoS Genet. 5, e1000760. http://dx.doi.org/10.1371/journal.pgen.1000760.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. http://dx.doi.org/10.1093/bioinformatics/btq461.

European Committee on Antimicrobial Susceptibility Testing, 2014. Data from the EUCAST MIC distribution website, accessed 11–26. http://www.eucast.org.

Fick, J., Söderström, H., Lindberg, R.H., Phan, C., Tysklind, M., Larsson, D.G.J., 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. Environ. Toxicol. Chem. 28, 2522–2527. http://dx.doi.org/10.1897/09-073.1.

Finley, R.L., Collignon, P., Larsson, D.G.J., McEwen, S.A., Li, X.-Z., Gaze, W.H., Reid-Smith, R., Timinouni, M., Graham, D.W., Topp, E., 2013. The scourge of antibiotic resistance: the important role of the environment. Clin. Infect. Dis. 57, 704–710. http://dx.doi.org/10.1093/cid/cit355.

Flach, C.-F., Johnning, A., Nilsson, I., Smalla, K., Kristiansson, E., Larsson, D.G.J., 2015. Isolation of novel IncA/C and IncN fluoroquinolone resistance plasmids from an antibiotic-polluted lake. J. Antimicrob. Chemother. 70, 2709–2717. http://dx.doi.org/10.1093/jac/dkv167.

Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression. 2nd ed. Sage, Thousand Oaks, CA.

Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzalez-Zorn, B., Barbé, J., Ploy, M.-C., Mazel, D., 2009. The SOS response controls integron recombination. Science 324, 1034. http://dx.doi.org/10.1126/science.1172914.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, e1002158. http://dx.doi.org/10.1371/journal.ppat.1002158.

Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. MBio 5. http://dx.doi.org/10.1128/mBio.01918-14.

Gutierrez, A., Laureti, L., Crussard, S., Abida, H., Rodríguez-Rojas, A., Blázquez, J., Baharoglu, Z., Mazel, D., Darfeuille, F., Vogel, J., Matic, I., 2013. β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. Nat. Commun. 4, 1610. http://dx.doi.org/10.1038/ncomms2607.

Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., Miller, S.I., 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436, 1171–1175. http://dx.doi.org/10.1038/nature03912.

Jernberg, C., Löfmark, S., Edlund, C., Jansson, J.K., 2007. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 1, 56–66. http://dx.doi.org/10.1038/ismej.2007.3.

Johnson, T.J., Singer, R.S., Isaacson, R.E., Danzeisen, J.L., Lang, K., Kobluk, K., Rivet, B., Borewicz, K., Frye, J.G., Englen, M., Anderson, J., Davies, P.R., 2015. In vivo transmission of an IncA/C plasmid in *Escherichia coli* depends on tetracycline concentration, and acquisition of the plasmid results in a variable cost of fitness. Appl. Environ. Microbiol. 81, 3561–3570. http://dx.doi.org/10.1128/AEM.04193-14.

Khan, G.A., Berglund, B., Khan, K.M., Lindgren, P.-E., Fick, J., 2013. Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities — a study in Pakistan. PLoS One 8, e62712. http://dx.doi.org/10.1371/journal.pone.0062712.

Kookana, R.S., Williams, M., Boxall, A.B.A., Larsson, D.G.J., Gaw, S., Choi, K., Yamamoto, H., Thatikonda, S., Zhu, Y.-G., Carriquiriborde, P., 2014. Potential ecological footprints of active pharmaceutical ingredients: an examination of risk factors in low-, middle- and high-income countries. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369. http://dx.doi.org/10.1098/rstb.2013.0586.

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H., Larsson, D.G.J., 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS One 6, e17038. http://dx.doi.org/10.1371/journal.pone.0017038.

Kümmerer, K., 2009. Antibiotics in the aquatic environment — a review — part II. Chemosphere 75, 435–441. http://dx.doi.org/10.1016/j.chemosphere.2008.12.006.

Larsson, D.G.J., 2014a. Pollution from drug manufacturing: review and perspectives. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369. http://dx.doi.org/10.1098/rstb.2013.0571.

Larsson, D.G.J., 2014b. Antibiotics in the environment. Ups. J. Med. Sci. 119, 108–112. http://dx.doi.org/10.3109/03009734.2014.896438.

Larsson, D.G.J., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J. Hazard. Mater. 148, 751–755. http://dx.doi.org/10.1016/j.jhazmat.2007.07.008.

Laurell, M., Norhstedt, P., Ryding, S.-O., 2014. Significance of Sustainability in Supply Chains on Behalf of SPP — A Pre-study (No. 2014:12), Konkurrensverket.se. Swedish Environmental Management Council.

Lewis, K., 2007. Persister cells, dormancy and infectious disease. Nat. Rev. Microbiol. 5, 48–56. http://dx.doi.org/10.1038/nrmicro1557.

Liu, A., Fong, A., Becket, E., Yuan, J., Tamae, C., Medrano, L., Maiz, M., Wahba, C., Lee, C., Lee, K., Tran, K.P., Yang, H., Hoffman, R.M., Salih, A., Miller, J.H., 2011. Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents. Antimicrob. Agents Chemother. 55, 1204–1210. http://dx.doi.org/10.1128/AAC.01182-10.

Liu, M., Zhang, Y., Yang, M., Tian, Z., Ren, L., Zhang, S., 2012. Abundance and distribution of tetracycline resistance genes and mobile elements in an oxytetracycline production wastewater treatment system. Environ. Sci. Technol. 46, 7551–7557. http://dx.doi.org/10.1021/es301145m.

López, E., Blázquez, J., 2009. Effect of subinhibitory concentrations of antibiotics on intrachromosomal homologous recombination in Escherichia coli. Antimicrob. Agents Chemother. 53, 3411–3415. http://dx.doi.org/10.1128/AAC.00358-09.

López, E., Elez, M., Matic, I., Blázquez, J., 2007. Antibiotic-mediated recombination: ciprofloxacin stimulates SOS-independent recombination of divergent sequences in Escherichia coli. Mol. Microbiol. 64, 83–93. http://dx.doi.org/10.1111/j.1365-2958.2007.05642.x.

Luo, Y., Xu, L., Rysz, M., Wang, Y., Zhang, H., Alvarez, P.J.J., 2011. Occurrence and transport of tetracycline, sulfonamide, quinolone, and macrolide antibiotics in the Haihe River Basin, China. Environ. Sci. Technol. 45, 1827–1833. http://dx.doi.org/10.1021/es104009s.

Martinez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321, 365–367. http://dx.doi.org/10.1126/science.1159483.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. Water Res. 47, 957–995. http://dx.doi.org/10.1016/j.watres.2012.11.027.

Morero, N.R., Monti, M.R., Argaraña, C.E., 2011. Effect of ciprofloxacin concentration on the frequency and nature of resistant mutants selected from *Pseudomonas aeruginosa* mutS and mutT hypermutators. Antimicrob. Agents Chemother. 55, 3668–3676. http://dx.doi.org/10.1128/AAC.01826-10.

Murray-Smith, R.J., Coombe, V.T., Grönlund, M.H., Waern, F., Baird, J.A., 2012. Managing emissions of active pharmaceutical ingredients from manufacturing facilities: an environmental quality standard approach. Integr. Environ. Assess. Manag. 8, 320–330. http://dx.doi.org/10.1002/ieam.1268.

O'Brien, T.F., 2002. Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clin. Infect. Dis. 34 (Suppl. 3), S78–S84. http://dx.doi.org/10.1086/340244.

Pal, C., Bengtsson-Palme, J., Rensing, C., Kristiansson, E., Larsson, D.G.J., 2014. BacMet: antibacterial biocide and metal resistance genes database. Nucleic Acids Res. 42, D737–D743. http://dx.doi.org/10.1093/nar/gkt1252.

Pruden, A., Larsson, D.G.J., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D.W., Lazorchak, J.M., Suzuki, S., Silley, P., Snape, J.R., Topp, E., Zhang, T., Zhu, Y.-G., 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ. Health Perspect. 121, 878–885. http://dx.doi.org/10.1289/ehp.1206446.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., Claverys, J.-P., 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science 313, 89–92. http://dx.doi.org/10.1126/science.1127912.

Quinlan, E.L., Nietch, C.T., Blocksom, K., Lazorchak, J.M., Batt, A.L., Griffiths, R., Klemm, D.J., 2011. Temporal dynamics of periphyton exposed to tetracycline in stream mesocosms. Environ. Sci. Technol. 45, 10684–10690. http://dx.doi.org/10.1021/es202004k.

R Development Core Team, 2011. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Relman, D.A., 2012. The human microbiome: ecosystem resilience and health. Nutr. Rev. 70 (Suppl. 1), S2–S9. http://dx.doi.org/10.1111/j.1753-4887.2012.00489.x.

Review on Antimicrobial Resistance, 2014. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. amr-review.org.

Rodríguez-Rojas, A., Rodríguez-Beltrán, J., Couce, A., Blázquez, J., 2013. Antibiotics and antibiotic resistance: a bitter fight against evolution. Int. J. Med. Microbiol. 303, 293–297. http://dx.doi.org/10.1016/j.ijmm.2013.02.004.

SPHS Secreteriat, UNDP Istanbul Regional Hub, 2015. 2014 Annual Report of the Informal Interagency Task Team on Sustainable Procurement in the Health Sector (SPHS), iiattsphs.files.wordpress.com. UNDP.

Swedish Medical Products Agency, 2009. Opportunities for Strengthening the Environmental Requirements Pertaining to the Manufacture of Medicinal Products and Active Pharmaceutical Ingredients in a National and International Context [in Swedish with English summary]. MPA (Swedish Medical Products Agency). MPA (Swedish Medical Products Agency).

Swedish Medical Products Agency, 2011. Underlag för att möjliggöra initieringen av en revidering av EU-lagstiftningen om god tillverkningssed, GMP, med syfte att lagstiftningen även ska omfatta miljöhänsyn [in Swedish], lakemedelsverket.se. MPA (Swedish Medical Products Agency).

Tello, A., Austin, B., Telfer, T.C., 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. Environ. Health Perspect. 120, 1100–1106. http://dx.doi.org/10.1289/ehp.1104650.

Thi, T.D., López, E., Rodríguez-Rojas, A., Rodríguez-Beltrán, J., Couce, A., Guelfo, J.R., Castañeda-García, A., Blázquez, J., 2011. Effect of recA inactivation on mutagenesis of *Escherichia coli* exposed to sublethal concentrations of antimicrobials. J. Antimicrob. Chemother. 66, 531–538. http://dx.doi.org/10.1093/jac/dkq496.

Torres-Barceló, C., Kojadinovic, M., Moxon, R., MacLean, R.C., 2015. The SOS response increases bacterial fitness, but not evolvability, under a sublethal dose of antibiotic. Proc. Biol. Sci. 282. http://dx.doi.org/10.1098/rspb.2015.0885.

Wang, J., Mao, D., Mu, Q., Luo, Y., 2015. Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants. Sci. Total Environ. 526, 366–373. http://dx.doi.org/10.1016/j.scitotenv.2015.05.046.

Warnes, G.R., Bolker, B., Gorjanc, G., Grothendieck, G., Korosec, A., Lumley, T., MacQueen, D., Magnusson, A., Rogers, J., 2013. gdata: Various R Programming Tools for Data Manipulation.

Wright, G.D., 2010. Antibiotic resistance in the environment: a link to the clinic? Curr. Opin. Microbiol. 13, 589–594. http://dx.doi.org/10.1016/j.mib.2010.08.005.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014. The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res. 42, D643–D648. http://dx.doi.org/10.1093/nar/gkt1209.

Zhang, Q., Lambert, G., Liao, D., Kim, H., Robin, K., Tung, C.-K., Pourmand, N., Austin, R.H., 2011. Acceleration of emergence of bacterial antibiotic resistance in connected microenvironments. Science 333, 1764–1767. http://dx.doi.org/10.1126/science.1208747.