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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:

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