World Health Organization

Background document to Guidance on wastewater and solid waste management for manufacturing of antibiotics

Evidence synthesis for deriving PNECs for resistance selection

Contents

Introduction

This document accompanies *Guidance on wastewater and solid waste management for manufacturing of antibiotics (WHO, 2024*) and provides additional information on derivation of PNECs for resistance selection as outlined in Box 3 of the guidance.

The foundation of antibiotic resistance development lies in the process of natural selection. Strains that develop increased resistance to a given antibiotic, either by changes in their own DNA, or by uptake of foreign DNA, or a combination of both, survive and multiply better than their more sensitive counterparts under a selection pressure by antibiotics. Consequently, exposure to antibiotics over time results in enrichment of resistant bacteria. In the unfortunate event that these strains cause infections, they are generally more challenging to treat. Numerous antibiotic resistance genes (ARGs) are often clustered on genetic elements like integrons, transposons, and plasmids. This clustering means that one class of antibiotics frequently co-selects for resistance to other classes. Antibiotics within the same class often share similar resistance mechanisms, inevitably leading to co-selection.

The primary concern with antibiotic pollution from antibiotic manufacturing is not how it might contribute to the spread of already widespread resistant strains circulating within society. Given that the number of manufacturing sites for a given antibiotic is relatively limited, such industrial pollution might, at most, incrementally contribute to other environmental and non-environmental transmission routes. Instead, a more pressing concern is whether it leads to the emergence of new, highly successful resistance genotypes, as this could have global repercussions [1]. The emergence of resistance can be accelerated by a selection pressure from antibiotics at various stages, including the processes when chromosomal ARGs acquire increased potential for intra- and inter-cellular mobility, selection of strains that have acquired ARGs horizontally, to the adaptation through compensatory mutations that reduce fitness costs [2].

Mutation-based resistance often develops relatively easily in the patient during therapy, without any necessary interaction with other bacteria. In contrast, the acquisition and emergence of mobile resistance in bacterial pathogens are expected to be significantly facilitated by the genetic diversity found in external environments, particularly in wastewater[3]. Therefore, it becomes crucial to reduce selection pressures in external environments as a precautionary measure to mitigate the risks associated with the emergence of new forms of resistance in pathogens that can subsequently spread to humans and animals.

Objectives

There are numerous methods available to determine the concentration at which an antibiotic does and does not have the ability to select for antibiotic resistant strains. Each of these approaches comes with its own advantages and disadvantages. The goals of this evidence synthesis are threefold:

1. To compile and critically assess various approaches for evaluating the antibiotic's potential to select for resistance in aquatic environments.
2. To propose a methodology for establishing Predicted No Effect Concentrations for resistance selection (PNECres) that can be applied to ensure the safe disposal of liquid waste generated during antibiotic manufacturing.
3. To provide a comprehensive list of PNECres values for different antibiotics, to be used for risk assessment within the framework of the current guideline.

LOECs, NOECs, PNECs, MSCs – which one to use?

he concepts of Lowest Observed Effect Concentration (LOEC), No Observed Effect Concentration (NOEC), and Predicted No Effect Concentrations (PNECs) are well-established in regulatory environmental toxicology. The LOEC represents the lowest concentration tested where a measurable effect significantly differs from the control group, while the NOEC is the highest tested concentration without a statistically significant difference to the control. In contrast to LOECs and NOECs (which are directly derived from the data at hand), the PNEC is an assessment taking into uncertainties not included in the executed experiment(s), for example that there might be alternative species or conditions that would be more sensitive than the tested one(s). Most often, the PNEC is derived from the NOEC by adding an assessment factor (see section below). For a given antibiotic, a specific test, and a particular endpoint, these three concentrations follow this order: LOEC > NOEC > PNEC.

In some selection studies, one may come across the concept of Minimal Selective Concentrations (MSCs). How does MSC relate to LOEC, NOEC, and PNEC in the context of resistance selection? MSC is similar to LOEC and NOEC in the sense that it is derived from the experimental data at hand. But in contrast to LOEC and NOEC, it is not based on an assessment of statistically significant effects, but is an estimate obtained by extrapolating competitive growth data across different test concentrations. It predicts the lowest concentration at which, theoretically, the selection of a resistant strain would begin to occur. In other words, MSC is the point where the cost for a bacterium to be resistant is estimated to be perfectly balanced by the antibiotic-induced growth reduction among its non-resistant competitors [4]. Unlike LOEC, representing a statistically confirmed effect level, the MSC represents an extrapolated concentration estimate where the fitness cost of the resistance is balanced by the antibiotic-conferred selection for the resistant mutant [5]. An advantage with MSCs is that it utilizes experimental data over several exposure concentrations (regression) whereas NOECs and LOECs are based only on replication within a given exposure concentration. Conversely, NOECs and LOECs are based on analyses of statistically significant effects, whereas MSCs are generally not. Notably, low replication per test concentration may lead to high LOEC and NOECs.

When working with the same dataset of experimental observations, the MSC will often be lower than the LOEC and the NOEC, but it can also be higher than both of these measures depending on various factors. If dose-response curves are steep, statistical power is high (due to good replication and low variability), and the tested concentrations are relatively close to each other, the differences between LOEC, NOEC, and MSC become small. In cases with high variance and limited replication, LOECs and NOECs tend to be higher. The estimate of the MSC can, in contrast to the LOEC and NOEC, fluctuate in any direction under such conditions. Particularly when assessing MSCs for resistance factors that aren't very costly for the bacterium to carry, there's a risk that the MSC estimate becomes infinitesimally small (or even negative) ([6]. MSC reporting often lacks a confidence interval, although it's theoretically feasible to generate one. Since determining an MSC doesn't depend on the observation of a significant effect at any exposure concentration, using MSCs without confidence intervals sometimes come with a risk of being overly cautious. When there is a choice between MSC data and PNEC data, this guidance will primarily rely on an approach based on statistical analyses and PNECs derived from LOECs/NOECs, given everything else alike. This is in line with recent recommendations [7] and follows the most common approach in environmental regulatory toxicology and risk assessment. In cases where comparable PNEC data is unavailable, MSC data can be a suitable replacement or complement.

Applying safety-factors (assessment factors) to generate PNECs

It is common practice in risk assessment to add a safety factor to account for remaining uncertainties, not covered in the experimental setup. Overall, it is difficult to know what a balanced assessment factor should be, essentially how precaution should be weighed against available evidence. In some applications, the magnitude of the safety factors depends on the availability of data. For example, if toxicity data is generated from just one type of organism, the applied safety factor is often larger than if toxicity data is generated from several organisms [7, 8]. Similarly, if a wide set of approaches have been used to generate data on selective potency, the risks for false negatives would be smaller, which could motivate a lower assessment factor. A non-flexible assessment factor may therefore contribute to systematic overprotection of more thoroughly investigated compounds (as the uncertainty is arguable smaller for such compounds). Large differences between test concentrations can also inflate the safety margin (increase the distance between the LOEC and the PNEC). Both 2-fold [9-11] and 10-fold [11-14] dilutions are common. With 10-fold dilution steps in test concentrations are applied, the derived PNEC becomes 100 times lower than the lowest concentration known to have an effect if an assessment factor of 10 is applied. One-hundred fold may be considered a rather large margin, particularly when multiple studies point towards a similar LOEC. Murray et al (2021) [7] discussed the use of assessment factor with regards to resistance selection. The authors proposed a flat safety factor of 10 from the NOEC to derive the PNEC, regardless of the design and endpoint used. This may be a pragmatic and simple compromise, but it comes with the setbacks described above.

For MSCs, Murray et al [7]) argued that no safety factor at all should be applied (as it is already an extrapolation). For some datasets, however, the MSC is higher than, or very close to the NOEC, meaning that a PNEC derived from the NOEC and an assessment factor of 10 will be much lower than the MSC derived from the same dataset. Also, from a conceptual perspective, there is a value in applying an assessment factor to generate “safe concentrations” also for MSC data to cover for e.g. other genes/species/contexts that are more sensitive than what the specific assay investigated. A flexible assessment factor specifically taking account suspected species differences in MSC data has been proposed by EFSA [15].

Minimal inhibitory concentrations (MICs)

Another frequently employed concept in selectivity assessment is the Minimal Inhibitory Concentration (MIC) of a particular strain to a specific antibiotic. MIC is typically based on growth data obtained through a standardized assay and represents the lowest antibiotic concentration that completely inhibits visible growth of the strain under defined conditions. It's important to note that selection of resistance often occurs at concentrations below, and sometimes well below, the MIC of the wildtype strain [5]. This happens because antibiotic exposure frequently leads to reduced growth of the susceptible wildtype strain, even at sub-MIC concentrations. As long as this growth reduction is greater than the reduction in growth of the resistant strains resulting from the increased fitness cost of being resistant, selection for resistance will take place. MIC data for a range of strains has been employed to estimate the potential for selection by various antibiotics, as described below.

Defining resistance

The term "resistance" requires some clarification, as there often is more or less wide spectrum in what antibiotic concentrations different bacterial strains from the same species can tolerate. In clinical terms, resistance refers to a bacterial strain's ability to survive antibiotic treatment at dosages typically used in patient therapy. These characteristics have been benchmarked against concentrations that can prevent visible bacterial growth in culture media, so that the ability to grow in culture media at a

defined breakpoint concentration corresponds reasonably well to the possibility to treat the infection. It's worth noting that clinical breakpoints primarily apply to bacteria causing disease. In simple terms, clinically, "resistant" means "untreatable" with a specific antibiotic. There's an alternative microbiological definition of resistance that can apply to any species, as it does not relate to treatability, but rather to any increased MIC over wildtype strains of the same species. Defining "wildtype" isn't always straightforward, making this definition challenging to apply universally. However, in cases where different bacterial strains are compared, it's often possible to conclude whether one is more or less resistant than another. In the context of assessing environmental selection and resistance evolution risks, any level of acquired resistance is a concern, not just resistance up to the clinical breakpoint. This is because environmental exposure levels are frequently much lower than clinical breakpoints, and resistance evolution is often a stepwise process. Therefore, in this context, we use the microbiological definition of resistance ([16].

Assays to assess the selective potency of antibiotic in aquatic environments

There is no formal, standardized assay for assessing the selective potency of antibiotics that is applicable to environmental settings. Various designs have been employed, each differing in multiple aspects. While this list may not encompass all possible variants, the following conceptual types, along with selected representative references, are outlined in Table 1. These assays vary in terms of design and readout, as discussed in more detail below and summarized in Table 1.

It's important to distinguish between selective concentrations on one hand from concentrations inducing resistance mutations or concentrations increasing the rate of horizontal transfer of mobile

Table 1. Conceptual comparison and overview of different assays and readouts to derive PNECs for antibiotic resistance selection in the environment.

biofilm/planctonic	planctonic	biofilm	biofilm	biofilm		planctonic	planctonic	planctonic	planctonic	planctonic	planctonic
Assay type: paired approach with isogenic pairs of bactera (**iso**), defined/natural comunity (**def/nat comm**): MIC-distributions of many strains and species (**many strains/species**)	iso	nat comm	nat comm	nat comm	nat comm	nat comm	nat comm	def comm	nat comm	nat comm	many strains and species
spontaneous **mutations**; 2) genetic (pcr of genes - **pcr**; metagenomics -**meta**); 3) phenotypic resistance (cell-sorting **cs**, plating with/without ab **nr/r, within** a species or **without** species differentiation) 4) total community growth **tot growth**; 5) diversity index **diversity** 6) 1% lowest **MIC**	cs, within	meta, diversity	pcr+meta	nr/r, within	n/r, without	nr/r,within	meta, diversity	nr/r,within	pcr	tot growth	MIC
Example of paper with above assay type/readout:	1	2	2	2	2	2	3	3	4	4	5
Conceptual benefits of assay:											
+ Assay taking into account community interactions	n	y	y	y	y	y	y	(y)	y	y	n
+ Assay addressing many resistance genes and genetic contexts in parallel	n	y	y	y	y	y	y	y	y	y	y
+ Assay that address selection potential in many species in parallel	n	y	y	n	y	n	y	n	y	y	y
+ Largely based on existing public data	n	n	n	n	n	n	n	n	n	n	y
+ Experimental parts based on standardised protocols	n	n	n	n	n	n	n	n	n	n	y
+ Low complexity of assay and analysis	n	n	n	n	n	(y)	n	(y)	y	y	(y)
+ Low costs	n	n	n	n	n	y	n	y	y	y	(y)
+ Approach already adopted by some stakeholders	n	n	n	n	n	n	n	n	n	n	y
+ PNECs already available for many antibiotics	n	n	n	n	n	n	n	n	n	n	y
+Assay unambigously demonstrating selection of resistance within species	y	n	n	y	n	y	n	y	n	n	n
+Assay with no risk that between-species selection confuses readout	y	n	n	y	n	y	n	y	n	n	y
+Assays with limited risks for reflecting effects on mutation rates instead of selection	y	y	y	y	y	y	y	y	y	y	y

Colours are added for visual clues. Green and pale-yellow colour indicate phenotypic or genotypic readouts, respectively. Please note that the classification of yes or no ("y" or "n") in some cases represent a simplification and can also be somewhat subjective. In some cases (y) is indicated to represent an intermediate level. References are included simply as examples: 1) Gullberg, E., et al., 2011. PLoS Pathog,7(7): p. e1002158. 2) Kraupner, N., et al., 2018. Environment International, 116: p. 255–268. 3) Kraupner, N., et al., 2021. Environment International,150: p. 106436. 4) Murray, A.K., et al., 2020. Environmental Health

resistance determinants on the other. Mutations and gene transfer events occur naturally, even in the absence of antibiotics, and various stressors can boost their rates. However, without antibiotic selection pressure, the genotypes resulting from these events are likely to fade rapidly [17]. Consequently, antibiotic-induced selection of strains with acquired resistance is considered the primary driver of resistance evolution. Assays assessing antibiotic effects on mutation and gene transfer rates are therefore not included here.

When selecting or recommending assays and strategies to define PNECs, it's crucial to consider both scientific and practical/regulatory aspects [18]. It's highly probable that no single assay will fulfil all requirements. Table 1 aims to categorize different assay types based on whether they meet various beneficial characteristics of an assay. Since fulfilment is often not a strict yes or no, but rather partial and contingent on specific circumstances, the classification reflects some degree of subjective judgment. Furthermore, certain characteristics may hold more significance than others; for instance, if guided by the precautionary principle, it's more crucial that an assay minimizes false negatives (setting a PNEC too high) rather than false positives (setting a PNEC too low). Conversely, if cost considerations are paramount, the opposite may hold true. If implementation challenges are a major concern, factors like low complexity, cost-efficiency, minimal infrastructure and specialist expertise requirements, and access to existing PNECs for a wide range of antibiotics become comparably more important.

Pairs of isogenic bacteria

A traditional and very clean but narrow method for studying selection involves competition experiments with two closely matched bacterial strains [5]. Ideally, these strains should differ only in their possession of a specific resistance factor, possibly accompanied by a marker for tracking purposes. This resistance factor could be a mutation in the native DNA of the strain, an acquired resistance gene, or the presence of an entire resistance plasmid. The readout of such experiments is the proportion of resistant versus non-resistant bacteria after multiple rounds of serial passaging. Sensitivity in terms of the ability identify differences in growth rate of resistant/non-resistant strains is very good (often down to 0.1% per generation). Enumerating resistant/non-resistant cells can be achieved through methods like cell-sorting using a tracking marker, often a fluorescent protein. This readout provides high reproducibility but in cases where the strains lack such markers, plating on media with and without antibiotics may be used. Assessing selection using pairwise competition of bacteria has limitations as it examines only one resistance factor at a time in a single context and species. A proposed way around the species limitation could be to factor in how the lowest MIC ([19]) of the studied species relate to the lowest MIC of the most sensitive species where such data is available [15]. Other resistance factors providing protection against the same antibiotic, or the same resistance factor in different genetic contexts can lead to varying cost-benefit ratios, which significantly affects the generated selective concentrations (often expressed as MSCs). Artificially introducing a resistance factor may also lead to issues like overexpression, a lack of compensatory mutations, and therefore higher carrying costs compared to naturally occurring resistance. This method also doesn't consider interactions with other bacteria of the same or different species.

Selection of spontaneous mutants during serial passaging

Another possibility to establish selective concentrations is to expose sensitive bacterial strains to different sub-MIC concentrations of antibiotics over many generations and at different timepoints evaluate the proportion of mutants that have acquired some level of resistance [5]. A challenge can be to distinguish between selection and induction of mutations, which is critical as the latter is not within

scope (see above). It is known that many antibiotics and other stressors can increase mutation rates, e.g. by inducing error-prone polymerase. However, without selection, mutans will not have an added survival value and the proportion of bacteria with an increased MIC will therefore not increase over time. Hence, including the criteria of increased resistance rates over time/generations may help disentangling selection from induction of mutants in these types of assays.

Complex communities

Studying complex communities offers several advantages over classical competition experiments involving just two strains. One key benefit is the diversity present in these communities, encompassing numerous strains with various resistance traits that operate differently in distinct contexts. These resistance traits also exhibit varying fitness levels under different exposure concentrations. The primary concern, particularly in the context of industrial emissions, is the potential for the promotion of evolutionary changes leading to the emergence of new mobile resistance gene types in pathogens. As these genes and their contexts are unknown, we also do not know anything about their fitness characteristics. Testing a variety of genes and contexts increases the likelihood of including resistance determinants with similar fitness characteristics to potentially successful but unknown resistance determinants.

Another advantage of studying complex communities is the consideration of interactions with multiple strains and species. These interactions can include protection through the production of antibiotic-degrading enzymes by other bacteria [9]. This mechanism doesn't alter the concentrations cells must be encountering in order to enable selection (i.e it does not influence the cells sensitivity to the antibiotic), but may reduce selection by reducing antibiotic levels around the cells. [20]. It is intuitive that extracellular enzymatic degradation through e.g. beta-lactam antibiotics can be important, but also intracellular antibiotic inactivation by other community members can provide protection [21]. The level of protection through antibiotic-degrading enzymes by other bacteria is probably highly context dependent and variable. To obtain an accurate MSC or PNEC relevant for comparison with measured effluent/environmental concentrations, quantifying the antibiotic within the community, not just relying on the nominal concentrations added, is necessary [9]. As concentrations may drop over time during exposure, measurements should ideally be done at the end of the exposure to align with the precautionary principle.

Community interactions can also directly impact fitness. In complex communities, more fit strains of other intrinsically resistant species, rather than somewhat less fit resistant strains of the same species, may occupy niches left open by the killing or reduction in the growth of sensitive strains of a given species. An example where this could have been the explanation is provided by Klumper et al. [22], where the MSC for aminoglycosides was over 10 times higher when isogenic bacterial pairs were embedded in a pig feces community compared to when studied as pairs. However, this MSC was based on nominal concentrations (as in many other studies), so it's possible that the actual exposure concentration was lower in the community context (overestimation of the MSC).

Considering both community interactions and the inclusion of a wide range of resistance determinants and contexts can make an assay more reflective of real-world scenarios. However, it's important to note that environmental communities vary, and typically only one or a few communities are tested to generate PNECs for a specific antibiotic under specific conditions. Therefore, even when using complex communities to generate PNECs, the results may not be directly applicable to different field situations.

Other challenges with complex natural communities are standardization difficulties, knowledge of the number of generations studied (and hence the selective strength, i.e. the the relative growth rate of

resistant versus susceptible strain, expressed per generation), and the possibility that they may not always contain a wide variety of strains with different resistance determinants and contexts, which provide the desired variability in fitness. These challenges can be partially addressed by using controlled mixtures of strains, although synthetic communities may not offer the same level of benefits in terms of interaction effects between bacteria compared to more complex, natural communities.

Another challenge in using communities in selection assays, especially environmental multi-species communities, is the strong dependence on culture conditions. The choice of media and temperature significantly affects which species and strains thrive and multiply. The more generations the community is cultured, the stronger the narrowing effect of the culture conditions, even if the inoculum is highly diverse. This can have several implications, such as limiting the ability to detect selective effects when culture conditions favour fast-growing Gram-negative bacteria while the tested antibiotic primarily affects Gram-positive bacteria and where the endpoint is sensitive to the relative proportion of sensitive bacteria e.g. total community growth [10].

Depending on the culture conditions, the resistant strains may either increase in absolute numbers over time during exposure (sometimes referred to as "positive selection") or decrease in in absolute number over time but increase in proportion to the sensitive strains . The concentration required to drive the latter has been referred to as the "minimal increased persistence concentration" [11]. Laboratory setups to derive PNECs are always, even when experiments are done in complex communities, very different from the reality that the lab experiment is intended to reflect. The culture media chosen, the oxygen tension, the temperature, the presence/absence of specific predators (e.g. phage, amoeba), the lack of heterogeneity of biotic and abiotic factors over time and space, the interactions with other bacterial members of the community as well as other factors will strongly determine which species and strains that will thrive or not. Most often, any lab conditions lead to a rapid loss or at least a very strong reduction of many or even most of the species in a complex community that added at the start of the experiment. Given the significant influence artificial culture conditions will have on the ability of species and strains to increase in absolute numbers over time, it becomes very shaky to make conclusions whether a given resistant strain or resistance factor (that is primarily hosted by certain species) will increase or decrease in absolute numbers over time in the real environment which the lab experiment is a model for. Hence, while the differentiation of positive selection and just increased persistence is valid when studied “in situ” in the environment of interest, it is difficult to draw conclusions about what will happen from lab experiments with communities. Therefore, the value of differentiating between “minimal selective concentrations” and “minimal increased persistence concentration” as derived from lab experiment with communities is very limited. For risk assessment, it is therefore essential to consider any concentration that leads to selection (whether positive or not).”

One may also talk about (lack of) positive selection in assays with single species. Here, the argument about influence of culture condition on the species’ relative fitness obviously has no relevance. Still, there are reasons to consider all detectable selection pressures for risk assessment, even if it is not sufficiently strong to favour the resistant strain over the non-resistant ones (positive selection) but only (significantly) diminishes the rate by which the sensitive strain(s) outcompetes the resistant one(s). The rational for that is that there are likely less costly resistant determinants out there that were not evaluated in the experiment. In the case of zero cost, any antibiotic-induced reduction of growth that affects the sensitive strain(s) more than the resistant one(s) would favour resistance development [1, 14].

Studying selection in planktonic bacteria vs. biofilms

Most assays typically investigate selection in free-living bacteria in a solution, also known as the planktonic phase. However, in nature, bacteria often thrive within biofilms. Biofilms are considered potential hotspots for the evolution of antibiotic resistance, primarily due to the close proximity of bacteria and the opportunities for horizontal gene transfer. On the flip side, biofilms offer an additional level of protection against antibiotic exposure. They achieve this through mechanisms like forming an extracellular matrix and having a lower cell turnover rate. These characteristics could potentially render biofilms less vulnerable to environmental antibiotic exposure ([23]. If this is indeed the case, there's a possibility that Predicted No-Effect Concentrations (PNECs) derived from biofilm assays may not adequately protect against selection among planktonic bacteria. The timing of antibiotic exposure, whether during biofilm formation or after the biofilm already has developed a protective matrix, could be a critical factor in this context.

Within- or between-species selection

Antibiotics in the external environment can lead to between-species selection, which may increase transmission risks for specific bacterial pathogens ([24]. However, the primary concern associated with antibiotic pollution from drug manufacturing is rather risks for the emergence and evolution of new, successful resistance genotypes [1]. Importantly, simple between-species selection does not provide an advantage to strains that have acquired resistance over those of the same species that have not. Therefore, within-species selection is the key driver for favouring the emergence and evolution of new forms of resistance. Evidence of between-species selection, however, can still provide information about the potential for within-species selection in species inhibited by the antibiotic. Measures of diversity that reflect the relative abundance of different taxa in a community post-exposure can indicate such a potential. Nevertheless, assays and endpoints that can identify within-species selection with certainty are more indicative of the risks associated with resistance evolution compared to those that cannot, from a conceptual point-of-view.

Measuring phenotypic resistance in one or many species

Several studies have assessed the selection of resistant bacteria in complex communities after exposure to varying antibiotic concentrations by counting colonies on solid media with or without antibiotics. When the media supports the growth of multiple species without clear differentiation, the readout cannot distinguish between changes in taxonomic composition and within-species selection. To overcome this challenge, highly selective media that permits the assessment of one species at a time can be employed [14]. For example, specific chromogenic agars can be used to accurately identify *E. coli* in complex sewage communities [25]. While this approach allows the assessment of within-species selection, it may overlook selection in other species that could be more sensitive to the studied antibiotic.

Measuring genes vs. phenotypic resistance

Numerous studies have used various assays to gauge the relative abundance of resistance genes (or genes often linked to resistance, like IntI1) as a measure of selection. An advantage of this approach is that it can capture selection processes in multiple species within a community simultaneously. On the downside, it may not account for resistance genes that aren't measured but still contribute to resistance in certain strains and species. Additionally, chromosomal resistance mutations are typically not considered unless amplicon sequencing is used [13]. This limitation is particularly relevant to PCR-based assessments of individual genes but to some extent also metagenomic-based, more open

approaches. Perhaps the most significant limitation is that it's often challenging, if not impossible, to definitively identify the host species of horizontally transferrable resistance genes using techniques like quantitative PCR or shotgun metagenomics. Despite many resistance genes having the ability to transfer between species, it's crucial to acknowledge that different species have distinct distributions of resistance genes and inherent levels of antibiotic tolerance (independent of acquired resistance). As a result, exposing a community to an antibiotic can lead to changes in taxonomic composition based on the inherent resistance levels of the species comprising the community. This can, as a straightforward consequence, impact the relative abundance of resistance genes, even in the absence of within-species selection for acquired resistance. This complicates the interpretability of mobile gene abundances as an indicator in selection experiments, as they at most can suggest within-species selection.

Total community growth as an endpoint

The use of total community growth as an endpoint has been suggested as a simplistic approach to assess the selective potency of antibiotics [10]. Reduced total growth implies that the antibiotic is inhibiting the growth of at least some species within the community. In this sense, reduced total growth hints at the potential for within-species selection, similar to diversity measures mentioned earlier. One significant limitation of total community growth as an endpoint is the high risk of false negatives and a very high Predicted No-Effect Concentration (PNEC). If the antibiotic only affects a small fraction of the tested community, there's a risk that even a strong effect on those (rare) bacteria may not significantly reduce total growth. Additionally, even if a larger part of the community is affected by the antibiotics, less sensitive species can seize the opportunity to fill the opened niche, utilize available nutrients, and compensate for the reduced growth in sensitive species or strains. As the assay focus on differences in cell density at the peak of growth rate, rather than cell density at the end of the assay, such compensatory growth would need to be fast for effects to remain undetected. Still, while a low PNEC based on total community growth indicates a potential for within-species selection, a high PNEC only weakly suggests that exposure concentrations below the PNEC are safe. Many other assays, including those based on parallel measurements of gene abundances in communities (metagenomics), have greater possibilities of detecting changes that only relate to a small portion of the tested community.

The MIC approach

The first idea of assessing risks for selection of antibiotic resistance in the environment using MIC data from clinical isolates was introduced by Kümmerer and Henninger (2003)[26] and later Tello et al (2012)[27]. In 2016, Bengtsson-Palme and Larsson [19] built on these concepts and proposed PNECres values for a very large range of antibiotics using extensive Minimum Inhibitory Concentration (MIC) data for primarily pathogenic bacteria available in the public EUCAST database. This database contains standardized, experimentally derived MIC data from thousands of strains for many antibiotics and up to 70 bacterial species. The underlying assumption for deriving PNECres values is that if a given concentration completely halts visible growth (MIC) of a sensitive strain of a species, a more resistant strain of that species would be selected for, regardless of the cost of carrying the resistance determinant, at least in some communities. However, this might not always occur under all conditions. Even when sensitive bacteria of a given species are inhibited by an antibiotic, a strain of the same species that carry a costly resistance determinant may not be favoured. This is because the resistant strain may be outcompeted by strains of other, intrinsically resistant species capable of filling the opened niche (if such strains exist in the community). In this case, an antibiotic concentration leading to inhibition of a given species may still not drive resistance development (no selection of resistant

strains over non-resistant strains of the same species) in that community. In this sense, a concentration that inhibits growth of a sensitive bacterium does not mean that within-species selection will take place in all communities exposed to that concentration. It rather indicates potential for within-species selection, similarly to endpoints in community assays that largely reflect reduced growth of some of the members (like total growth, or diversity measures). The approach is based on the 1% lowest MIC value in the most sensitive species, rounded down to the nearest 2-fold dilution, as used by EUCAST. However, as MIC data is sometimes available for only a few species, the 1% lowest MIC (and hence the PNEC) may be higher for antibiotics with limited species-specific data. Therefore, the approach adjusts the 1% lowest MIC value to account for data availability. It also adds a flat 10-fold assessment factor to accommodate the expected difference between the MIC and the presumed Minimum Selective Concentration (MSC). This is arguable a simplification, as the difference between MIC and the MSC is likely to vary across different combinations of antibiotics, species, resistance factors and genetic contexts. The resulting value is referred to as the PNECres. PNECs derived using the MIC approach have been widely used for risk assessments in the scientific literature since its publication. It has also been adopted by the AMR alliance ([28, 29] and included in a law proposal in India ([30].

Potential refinements

Costs associated with carrying an unknown resistance determinant are unclear. Based on experience from studying known resistance factors, these costs can be very small, especially after compensatory mutations have occurred [4]. From a precautionary principle standpoint (worst-case scenario), one could thus argue that costs should be considered zero [1, 14]). This implies that the lowest antibiotic concentration that significantly reduces the growth rate of a sensitive strain of a given species has the potential to select for resistance in that species. This approach bears some similarity to the MIC approach described earlier, but it bases the assessment of risks on concentrations on the lower, less-inhibited end of the dose-response curve, rather than on the MIC. Therefore, from a conceptual perspective, the "no-cost" approach is the most conservative and protective. Similar ideas have been explored by Greenfield et al. [31], who have considered refining selective concentration derivations from the MIC approach by accounting for the shape of dose-response curves and how far below the MIC a growth reduction can be expected. However, while MIC data is widely available, there is limited public data on antibiotic concentrations that start to affect bacterial growth (LOECs and NOECs for growth) in different strains and species, making this approach challenging, at least in the short term. The "no-cost" approach remains relatively unexplored but may become an alternative for consideration in the future.

If the "no-cost" approach is applied at the community level, it could be interpreted that any concentration affecting the taxonomic distribution might potentially lead to resistance. However, this interpretation may not always be straightforward, as antibiotics not only act as toxicants to bacteria but can also serve as food for some species [32] or possibly act as signalling molecules [33], both of which can result in changes in community composition. Resistance is only advantageous from the standpoint of antibiotics acting as toxicants. Particularly when changes in community composition are limited, it can be challenging to determine whether changes in taxonomic distribution/diversity indexes are the result of antibiotics acting as toxicants or through other mechanisms.

Mixture effects

While most environmental regulatory guidelines focus on pollutants individually, exposure in environmental media is typically complex. Ideally, interaction effects between different antibiotics and between antibiotics and other constituents of the environmental media should be considered.

Interactions can be additive, antagonistic, or synergistic. Additive effects are generally expected between antibiotics from the same class, and often also between antibiotics from different classes. Consequently, assessing risks with residual antibiotics individually, as in the present guideline, may underestimate the actual risks. However, evaluating interaction effects can be challenging due to variations in wastewater composition, often with many unknown constituents, limiting predictive approaches [34]. One way to address this limitation is to assess the selective ability of whole effluents, as done by Kraupner et al. [35] for hospital wastewater. While promising, the testing of whole effluents for selective potency still requires further development and benchmarking before it can be widely implemented in regulatory guidelines.

Influence of other experimental variables

While pros and cons are listed for each of the listed conceptual approaches, there are certainly other variables that contributes to how sensitive a given setup would be. This relates for example to the length of exposure, temperature, choice of media, the bacterial genes/species/strains/communities used in the assay and the detection method used for the readout, whether genotypic or phenotypic. Hence, even assays that apply the same conceptual approach may differ substantially in sensitivity. It is unlikely that any setup is optimised for sensitivity on all aspects.

Comparison of effect concentrations derived by different methods

Murray et al [7] recently compiled experimental LOECs, MSCs and PNECS for other methods than the MIC-approach. There were only such data for 11 antibiotics, whereas there are publicly available MIC-derived PNEC data for 100 antibiotics (antifungals and combinations removed from the list by [19]). For many of these 11 antibiotics, differences in derived selective potency between studies were large, more than 100-fold in several cases. For erythromycin, the highest listed PNEC (12,500µg/L) is a mis-citation, but even the correct value (1,250µg/L) is very high. This value is generated by the SELECT method with growth as endpoint (Murray et al 2020) and is 1,250 times higher than the PNEC based on the MIC-approach (1µg/L). In this case, the SELECT approach is very likely to underestimate the selective potency for the reasons presented earlier, including that the medium used do not favour Gram-positive bacteria, the main target of erythromycin. In fact, the LOEC in the SELECT growth assay was 25,000µg/L [10]. Fifty-nine of the bacterial species covered in EUCAST have a most common MIC (the mode) that is below the SELECT PNEC value for erythromycin. ECOFF refers to the concentration that completely stops growth of all "wild-type" strains of the species, i.e. those that are not considered to have acquired resistance. It is therefore highly unlikely that 1,250µg/L would be protective for resistance selection for erythromycin. The second highest value is an MSC value of 3000µg/L referring to [6]. It says in [7] that it is based on isogenic pairs of *E coli* with an introduced *mph*-operon. However, this is again a mis-citation, as 3000µg/L refers to carriage of a large and costly resistance plasmid, not just the *mph*-operon. According to the original reference, the MSC based on a strain with a chromosomally introduced *mph*-operon was estimated to less than 200µg/L, and the authors stressed that the estimate was uncertain as costs for carrying the *mph*-operon was very low and they did not experimentally test lower concentrations than 250µg/L [6]. Finally, Stanton et al [11] reported an MSC of 514.1µg/L and a PNEC of 50µg/L based on relative *ermF* abundance in a community setup. Still, the MIC-derived PNEC is clearly lower than other estimates, raising the concern that it is overprotective. It is based on the most sensitive species in the EUCAST database, in the case of erythromycin it is *Corynebacterium diphtherie*, the bacterium causing the disease diphteria. The lowest reported MIC is 4µg/L while 8µg/L is sufficient to completely stop growth of most strains of this species according to the EUCAST database. Although *Corynebacterium diphtherie* primarily live in and on humans, other members of the same genus thrive in water. However, as they often have rather special requirements

in order to be cultivable, it is unlikely that a selection of species within the *Corynebacterium* genus would be detected by the assays used. In light of this, a PNEC of 1µg/L does not appear overprotective. Similar comparisons can be made for other antibiotics for which differences between methods are large (e.g. azithromycin, kanamycin; [7, 19]. In none of these cases are there good reasons to dismiss the lower PNEC over the reported higher PNECs.

There are three antibiotics (ciprofloxacin, tetracycline and trimethoprim) where other methods than the MIC-approach have generated the lowest PNECs available. In addition, Murray et al [7] also reported a lower PNEC for streptomycin (1µg/L), but that is a mis-citation from the original reference (1mg/L; [5]. It is noteworthy that these three are the antibiotics that are most extensively studied for their selection potential in the environment. It is therefore quite plausible that if other antibiotics were equally extensively investigated, it would in many cases lead to lower PNECs than those currently derived using MIC-data.

Ciprofloxacin is probably the antibiotic that has been studied the most for its environmental selection potential, using different setups and endpoints. The MSC derived from competition experiments of isogenic pairs of E coli carrying a low-cost chromosomal mutation is. 0.1µg/L [9]. Using the provided raw-data, a re-calculated NOEC and LOEC of selection coefficients from these isogenic competition experiment would be 0.23µg/L and 0.46µg/L, respectively, using ANOVA with Dunnett's posthoc-testing (one-sided) against the control group (alfa level 0.05). This way of comparing selection coefficients to those of the control group would correspond to defining the MIPC according to Murray et al (2018). Kraupner et al [13] reports a LOEC of 1µg and a NOEC of 0.1µg/L for qnrD selection in complex biofilms. Murray et al [10] reports a LOEC of 0.98µg/L, a NOEC of 0.5µg/L and a PNEC of 0.05 as derived from an experiment with sewage community and total community growth as endpoint. Stanton et al [11] reported an MSC of 10.77µg/L and a LOEC of 15.6µg/L based on relative IntI1-abundance in a complex community exposed to different concentrations of ciprofloxacin. The derivation of the MSC is a bit unusual (based on a fourth-order polynomial regression) that crosses the Y-axis at a positive value, which would imply selection (positive selection coefficient) already without any antibiotic at all. This is however, not considered, but only a point where the selection coefficient (after dropping below zero with increasing concentrations) becomes positive a second time is used to derive the MSC. Also, the LOEC is derived using an alfa level of 0.10 (rather than more standard 0.05). Had the alfa level been 0.05, the LOEC would have been 62.5µg/L (NOEC 31.25µg/L, PNEC 3.125µg/L using the applied Gamma GLM analyses. Note that with ANOVA and Dunnett's posthoc test, the LOEC would have been 125µg/L. Murray et al [7] do report an exceptionally low MSC from another study by Vos et al (0.004µg/L) but is a mis-citation as the original reference states 4µg/L [34]. As the authors did not include a treatment group without ciprofloxacin, the LOEC can formally not be calculated, but based on the provided data it is likely to be close to the MSC and certainly not higher than 6.25µg/L. The 1% lowest MIC (an endpoint than in some sense can be treated as a LOEC) from the EUCAST data is 2µg/L (which is the lowest concentration tested), but an extrapolation of the distribution of reported MICs predicts a lowest MIC of 1µg/L. There is a recent study comparing the growth of a sensitive and a ciprofloxacin-resistant strain of *Neisseria gonorrhoeae* under different ciprofloxacin concentrations [36]. They report a very low MSC (0.007µg/L). While N. gonorrhoeae indeed is one of most sensitive species to ciprofloxacin listed in EUCAST, the methodology to derive the MSC from generated data is flawed, and very different from the approach described by e.g. Gullberg et al (2011)[5]; From a plot of the exposure concentration versus growth (rate or AUC) of the sensitive strain and resistant mutant, the MSC was defined as the average of three experiments with no internal technical replication, leading to three sets of highly non-monotonous experimental growth data. In addition, no curve fitting was used to compensate for variability, but the MSC was defined as the concentration where a straight line drawn between each of the test concentration (creating something similar to a zig-zag pattern)

crossed the zig-zag line for the other strain. With this approach, the individual MSC estimates varied almost two orders of magnitude between replicates, reflecting the uncertainty of the overall MSC estimate. MSC data from this study were therefore not taken into account.

It is noteworthy that the three lowest credible LOECs which are the ones reported by Kraupner, Gullberg and Murray et al, using completely different setups and endpoints, as well as the lowest reported MIC, do not differ more than ca two-fold. This provides relatively strong support that 0.46-1µg/L reasonably well reflects the lowest concentration that can select for resistance in different situations. That the LOEC is higher based on some other studies is not surprising given the many reasons why an assay may not be the most sensitive one in a given situation. The selective potency of tetracycline has also been investigated in several studies. The LOEC for relative *tetG* abundance in sewage microcosms was reported as 1µg/L, [11] although this was based on an alfa-level of 0.10 rather than 0.05. With 0.05 as alfa-level a revised LOEC for *tetG* in sewage microcosms would be 10µg/L, the NOEC 1µg/L and the PNEC 0.1µg/L. In experiments with sewage biofilms, the LOEC of *tetA* ($p<0.01$) and tetG ($p>0.05$) was 1µg/L [12]. Notably, 1µg/L was the lowest concentration tested in the biofilm experiments, hence a NOECs or PNECs were not established. It should also be noted that in this paper the term MSC was incorrectly used, but it really refers to the LOEC. Competition experiments with isogenic pairs of bacteria have resulted in somewhat higher MSCs of 15µg/L [37] and 45µg/L [5]. The lowest MIC observed in EUCAST (66 species) is 16µg/L and the corresponding MIC-derived PNEC is 1µg/L [19]. Quinlan et al (2011)[37] exposed freshwater stream mesocosms to tetracycline at different concentrations and reported significant effects on several endpoints at the lowest concentration tested (0.5µg/L) including bacterial cells per surface area, percent filamentous cyanobacteria and the relative percent tetracycline resistant bacteria (without species resolution). However, due to an intricate setup there were no replication of the mesocosms (n=1) and one cannot exclude that significant results are due to pseudo-replication (only technical replicates used for statistical analyses). Also, the percent tetracycline resistant bacteria showed no dose-response pattern and the only significant concentration was the lowest one tested.

Another well-studied antibiotic is trimethoprim, with LOECs from several studies ranging within a relatively small interval from 31.25µg/L to 100µg/L according to [7]. However, the LOECs (for persistence) of introduced *dfr*-genes from [14] were not included (10µg/L) in this summary. The lowest reported MIC from EUCAST is 16µg/L with a corresponding PNEC of 0.5µg/L [38].

Strategy for establishing PNECs for resistance selection

As previously outlined, the methods proposed for defining Predicted No-Effect Concentrations (PNECs) each have their advantages and disadvantages, and there's no universally accepted standard for setting PNECs. Hence, the precautionary principle plays a vital role in the overall strategy, at the same time bearing in mind risks and consequences for setting overly stringent criteria. PNECs are determined individually without considering potential mixture effects. The rationale for setting PNECs in this guidance is to select the lowest PNEC for a given antibiotic that does not appear to be overly protective on good grounds, alternatively carry a risk of greatly underestimating selection risks.

PNECs based solely on experimental data from setups that do not address selection potential in many species and/or do not address many resistance genes and contexts (see table 1) has an inherent risk of being under-protective (e.g. by only investigating a particularly insensitive species, a costly resistance factor, or a costly genetic context). Similar risks are present for PNECs based solely on assays where a surrogate endpoint for selective effects is easily masked or compensated for (such as total growth of communities[10]). When deriving PNECs, one shall always be aware of risks for false negatives and make thorough efforts to minimize such risks in the choices of experimental design and

endpoints. In the case any company or organization with economic interests in the outcome generates PNECs with potential for later inclusion in the guidance, they should pay particularly close attention to such risks. Therefore, if there are good reasons to believe all available PNECs are grossly under-protective, the default value of 50 ng/L should still apply [39]. An important reflection here is that MIC-derived PNECs based on EUCAST data (which has its apparent limitations but does not appear to suffer from these specific shortcomings) exists for a very wide set of antibiotics, alleviating the need for applying the default PNEC for those.

In addition to conceptual limitations as raised above, there are numerous other reasons to why a PNEC may not be trusted, including low quality of the underlying research. While strategies for assessing the quality of published research exist ([40], these tend to focus on accurate reporting and may be less sensitive to specific shortcomings in assay design or interpretation. Therefore, the WHO, as the guidance owner, should establish a permanent group of international, independent experts in antibiotic resistance selection, bacterial evolution, environmental pollution, and regulatory affairs. This group should regularly review the list of PNECs based on current knowledge and their expert judgement. If necessary, this external group should also have the authority to propose criteria revisions for PNECs, subject to approval by the WHO.

The approach that currently provide data for the highest number of antibiotics by far is the MIC approach. Therefore, at least for the time being, PNECs derived from this approach is the primary drivers for the PNECs in the current guidance document. The MIC approach aligns reasonably well with the listed criteria in Table 1. However, it's important to note that the MIC approach doesn't directly assess selection but predicts it from empirical data on growth. Additionally, it doesn't account for community interactions. For certain antibiotics, it provides lower PNECs than other approaches, leading to concerns of potential over-protection. On the other hand, it's not straightforward to conclude that alternative methods resulting in individual, higher PNECs are more accurate in reflecting the "real" environmental selection risk. The decision to use the lowest available PNEC or consider a higher PNEC should be made by the expert committee once established.

PNECres data exists for over 100 antibiotics, which likely covers the overwhelming majority of antibiotics produced globally, as measured either by volume or value. Still, there are numerous antibiotics (and active intermediaries that are less explored) for which no reliable PNECs for resistance selection are available. In such cases, a default value of 50 ng/L will be applied [39]. The WHO welcomes proposals for new or refined PNEC values, including supporting material, from anyone, including industries producing the antibiotics. These proposals will be reviewed by the expert group during their annual meetings for inclusion in the list of PNECs. There is also an option to seek advice from the WHO on the strategy to generate PNEC data for a specific substance, with requests to be submitted at least three months before an annual meeting.

In this guidance, and as recommended earlier [7] an assessment factor of 10 is applied unless it is identified that the underlying experimental evidence is sufficiently large to make an exception as the standard approach may otherwise be overprotective (see also sections on “Applying safety-factors (assessment factors) to generate PNECs”, “Comparison between PNECs derived by different methods”, and “List of PNECs for resistance selection”).

Transparency requirements for PNEC generation

To ensure the reliability of PNEC values used in the guideline, transparency regarding the methodology for determining these values is essential. Ideally, full transparency concerning the underlying data should also be provided. Since there is no universally accepted standard for deriving PNECs for

resistance selection, a reference to the methodology described in a public document is the absolute minimum requirement. The expert committee will then assess whether the PNEC value should be used. Without transparency regarding methodology, the default PNEC value of 50 ng/L [39] will be applied in the guideline until an alternative PNEC is determined by the expert committee.

List of PNECs for resistance selection

Table 2 lists the original sources for each PNEC value, in most cases, originating from PNECs derived by Bengtsson-Palme and Larsson (2016) [19] using the MIC approach. Only PNECs for individual substances (not combinations) are included. Some substances in the original dataset were antifungals or beta-lactamase inhibitors and have been excluded. All "PNEC-MIC" data listed by the AMR Alliance (as of February 22, 2023) except the PNEC-MIC for framycetine (60ng/L) are based on the Bengtsson-Palme and Larsson (2016) dataset [19]. As there is no background data available behind the derivation of the framycetine PNEC-MIC, the default PNEC (50ng/L) will apply here.

Despite that Kraupner et al reported the highest LOEC of the three lowest credible LOECs for ciprofloxacin (0.46-1µg/L), they applied larger (10-fold versus 2-fold) dilution steps, leading to a lower NOEC and PNEC than the other two studies. This represents a rather large margin, given the extent to which ciprofloxacin has been investigated and the coherence of the lowest LOEC across studies. The MIC-derived PNEC (0.064µg/L [13]) the PNEC from the Murray et al study (0.05µg/L) and a recalculated PNEC from Gullberg et al (0.023µg/L) are all higher. Basing the PNEC for the guidance on the study providing the lowest LOEC appears to provide a sufficient safety margin in a case like this. This suggests that the recalculated PNEC from Gullberg et al, rather than the one from Kraupner et al, would be used as an overall PNEC for resistance selection for ciprofloxacin in this guidance.

There is not a similar clustering of the lowest reliable LOECs for tetracycline as for ciprofloxacin. The lowest reliable LOEC is 1µg/L (with no lower concentration tested; [12]) and the lowest NOEC (as derived using an alfa level of 0.05) is also 1µg/L (revised here, data from [11]), leading to a PNEC of 0.1µg/L that is proposed to be used here. This is ten times lower than the MIC-based PNEC for tetracycline [38].

For trimethoprim, the study reporting the lowest LOEC applied 10-fold dilution factors, rather than 2-fold [14]. This leads to a NOEC of 1µg/L and applying a standard assessment factor a corresponding PNEC of just 0.1µg/L. Given the clustering of all of the lowest LOECs between 10 and 100 from several studies, it may be overly protective to set the PNEC in the guidance to 0.1µg/L for trimethoprim. Reverting to the somewhat higher PNEC based on the MIC-approach (0.5µg/L) is therefore suggested here.

Table 2. List of PNECs for resistance selection

API	PNECres (µg/L)	Ref.
Amikacin	16	1
Amoxicillin	0.25	1
Ampicillin	0.25	1
Avilamycin	8	1
Azithromycin	0.25	1
Aztreonam	0.5	1
Bacitracin	8	1
Benzylpenicillin	0.25	1
Capreomycin	2	1
Cefaclor	0.5	1
Cefadroxil	2	1
Cefaloridine	4	1
Cefalothin	2	1
Cefazolin	1	1
Cefdinir	0.25	1
Cefepime	0.5	1
Cefixime	0.06	1
Cefoperazone	0.5	1
Cefotaxime	0.13	1
Cefoxitin	8	1
Cefpirome	0.06	1
Cefpodoxime	0.25	1
Ceftaroline	0.06	1
Ceftazidime	0.5	1
Ceftibuten	0.25	1
Ceftiofur	0.06	1
Ceftobiprole	0.25	1
Ceftriaxone	0.03	1
Cefuroxime	0.5	1
Cephalexin	4	1
Chloramphenicol	8	1
Ciprofloxacin	0.023	2
Clarithromycin	0.25	1
Clinafloxacin	0.5	1
Clindamycin	1	1
Cloxacillin	0.13	1
Colistin (Polymyxin E)	2	1

API	PNECres (µg/L)	Ref.
Daptomycin	1	1
Doripenem	0.13	1
Doxycycline	2	1
Enrofloxacin	0.06	1
Ertapenem	0.13	1
Erythromycin	1	1
Ethambutol	2	1
Faropenem	0.02	1
Fidaxomicin	0.02	1
Florfenicol	2	1
Flumequine	0.25	1
Fosfomycin	2	1
Fusidic acid	0.5	1
Gatifloxacin	0.13	1
Gemifloxacin	0.06	1
Gentamicin	1	1
Imipenem	0.13	1
Isoniazid	0.13	1
Kanamycin	2	1
Levofloxacin	0.25	1
Lincomycin	2	1
Linezolid	8	1
Loracarbef	2	1
Mecillinam	1	1
Meropenem	0.06	1
Metronidazole	0.13	1
Minocycline	1	1
Moxifloxacin	0.13	1
Mupirocin	0.25	1
Nalidixic acid	16	1
Narasin	0.5	1
Neomycin	2	1
Netilmicin	0.5	1
Nitrofurantoin	64	1
Norfloxacin	0.5	1
Ofloxacin	0.5	1
Oxacillin	1	1

API	PNECres (µg/L)	Ref.
Oxytetracycline	0.5	1
Pefloxacin	8	1
Phenoxymethyl-penicillin	0.06	1
Piperacillin	0.5	1
Retapamulin	0.06	1
Rifampicin	0.06	1
Roxithromycin	1	1
Secnidazole	1	1
Sparfloxacin	0.06	1
Spectinomycin	32	1
Spiramycin	0.5	1
Streptomycin	16	1
Sulfamethoxazole	16	1
Teicoplanin	0.5	1

API	PNECres (µg/L)	Ref.
Telithromycin	0.06	1
Tetracycline	0.1	3
Thiamphenicol	1	1
Tiamulin	1	1
Ticarcillin	8	1
Tigecycline	1	1
Tilmicosin	1	1
Tobramycin	1	1
Trimethoprim	0.5	1
Trovafloxacin	0.03	1
Tylosin	4	1
Vancomycin	8	1
Viomycin	2	1
Virginiamycin	2	1

Table 2 references:

1) Bengtsson-Palme, J. and D.G.J. Larsson, Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environment International, 2016. 86: p. 140–149;

2) Gullberg, E., et al., Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog, 2011. 7(7): p. e1002158. (effect data recalculated as described above);

3) Stanton, I.C., et al., Evolution of antibiotic resistance at low antibiotic concentrations including selection below the minimal selective concentration. Communications Biology, 2020. 3(1). (effect data recalculated as described above).

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