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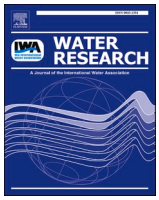

Review

A critical meta-analysis of predicted no effect concentrations for antimicrobial resistance selection in the environment

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ABSTRACT

Antimicrobial resistance (AMR) is one of the greatest threats to human health with a growing body of evidence demonstrating that selection for AMR can occur at environmental antimicrobial concentrations. Understanding the concentrations at which selection for resistance may occur is critical to help inform environmental risk assessments and highlight where mitigation strategies are required. A variety of experimental and data approaches have been used to determine these concentrations. However, there is minimal standardisation of existing approaches and no consensus on the relative merits of different methods. We conducted a semi-systematic literature review to collect and critically appraise available minimal selective concentration (MSC) and predicted no effect concentration for resistance (PNECR) data and the approaches used to derive them. There were 21 relevant articles providing 331 selective concentrations, ranging from 0.00087 μg/L (ciprofloxacin) to 2000 μg/L (carbenicillin). Meta-analyses of these data found that selective concentrations are highly compound-dependent, and only a subset of all antimicrobials have been the focus of most of the research. The variety of approaches that have been used, knowledge gaps and future research priorities were identified, as well as recommendations for those considering the selective risks of antimicrobials in the environment.

1. Introduction

1.1. Antimicrobial resistance and AMR in the environment

Antimicrobial resistance (AMR) is the process by which microorganisms (bacteria, fungi, viruses, parasites) acquire the ability to resist exposure to antimicrobials (WHO, 2015). AMR infections are one of the leading causes of death worldwide, with over one million deaths being directly attributable to antibacterial resistance in 2019 (Murray et al., 2022). By 2050, AMR infections are predicted to cause an estimated 10 million deaths each year, as well as a 100 trillion US Dollars loss in Gross Domestic Product (O'Neill, 2016). The One Health approach recognises that the health of humans and animals are interconnected with, and impacted by, the environment (HMGovernment, 2019; WHO, 2015). Yet the environment is often overlooked, despite being of concern in terms of its contribution to the origins, amplification, persistence and dissemination of AMR, and transmission of resistant pathogens and mobile resistance genes via environmental exposure.

The use and often overuse or misuse of antimicrobials in human and veterinary medicine, as well as in personal care products (Boxall et al., 2012), plant protection products and disinfectants (Murray et al., 2024), provides a multitude of pathways by which they can enter the environment (Fig. 1). The concentrations of antimicrobials reaching the environment depend on how they are manufactured, used, and disposed of. In addition, factors such as human/animal metabolism, (bio)degradability, chemical properties of the antimicrobial and dilution in the receiving environment all influence environmental concentrations. Many environments contaminated with antimicrobials are also polluted with AMR organisms, which provides opportunities for the enrichment of resistant human, animal and/or plant pathogens. There are also opportunities for horizontal gene transfer, and subsequent selection, of resistance mechanisms from environmental to human, animal or plant associated bacteria (and *vice versa*) (Larsson and Flach, 2021).

One approach to understanding the risks posed by antimicrobials in

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the environment is to perform an environmental risk assessment based on their potential to increase or "select for" AMR at measured environmental concentrations (MECs) or predicted environmental concentrations (PECs) (Murray et al., 2021). This approach requires data on threshold concentrations, i.e., concentrations which are unlikely to select for AMR. Once these selective concentrations are generated, these can be compared to the MEC or PEC of a specific environment to understand whether there is a risk of selection in such environments (e.g., Hayes et al. (2022), Read et al. (2024) and Mortimer et al. (2020)). Currently, selective concentration data are scarce, partly because this is an emerging area of science and there is no standardized, widely accepted method to determine the lowest concentration of an antimicrobial that increases AMR. Although a growing body of research in this area exists, several different approaches, including MIC-based methodologies (e.g., Bengtsson-Palme and Larsson (2016); Zhang et al. (2022)) and experimental systems (e.g., Gullberg et al. (2011); Kraupner et al. (2020); Murray et al. (2020)), have been used, as well as different focal species/communities tested, analytical tools, and statistical methods. These differences complicate comparisons across studies. This review will collate and discuss the findings of these and other studies in more detail below, evaluate the various approaches used and provide clarifications regarding the terminology used in this research space.

Previous work has collated and assessed this type of data before. For example, Murray et al. (2021) published a narrative review providing an overview of selective endpoints and focused on integrating these into current environmental risk assessments. Similarly, in 2023, the WHO published a draft paper on deriving predicted no effect concentration for resistance (PNECRs) in the context of pharmaceutical manufacturing waste management. As with Murray et al. (2021), this was a narrative review of the data (WHO 2023). To the best of the authors' knowledge, there are currently no publications assessing these studies using semi-systematic searching strategies or undertaking a meta-analysis on PNECR data.

1.2. Terms and definitions used in this study

Within this area of research, several terms referring to selective concentrations/thresholds have been used. To some degree, these terms have been used interchangeably in the literature, although each has a distinct meaning, which are outlined in Table 1.

Minimal Selective Concentrations (MSCs) are often reported as selective thresholds but some studies have taken this a step further and applied Assessment Factors (AFs) to MSCs, to generate PNECRs. PNECRs have been determined in a variety of ways, and sometimes are just referred to as Predicted No Effect Concentrations (PNECs, e.g., Kraupner et al. (2020)). Throughout this study, we will use the PNECR term, even where the original study reported the value as a PNEC.

Some PNECRs have been estimated from existing data sources, namely Minimum Inhibitory Concentration (MIC) datasets. One approach estimated PNECRs by applying an AF of 10 to MIC data to account for the difference between 'size-adjusted, lowest' MICs of susceptible strains and the MSC, for many antibiotics (Bengtsson-Palme and Larsson, 2016). These PNECRs have been partially adopted by the AMR Industry Alliance in their recommendations for threshold antibiotic concentrations in the receiving water for use in the assessment of pharmaceutical manufacturing effluent (AMRIA, 2023; Tell et al., 2019). Some PNECRs have also been determined from experimental evolution studies using communities of single species of bacteria (e.g., Kraupner et al. (2020)) and complex communities of bacteria (e.g., Murray et al. (2020)). In experimental studies, AFs are usually applied to NOECs to generate PNECRs. So far, there has been little discussion around the usage of different AFs in the AMR context, with most studies

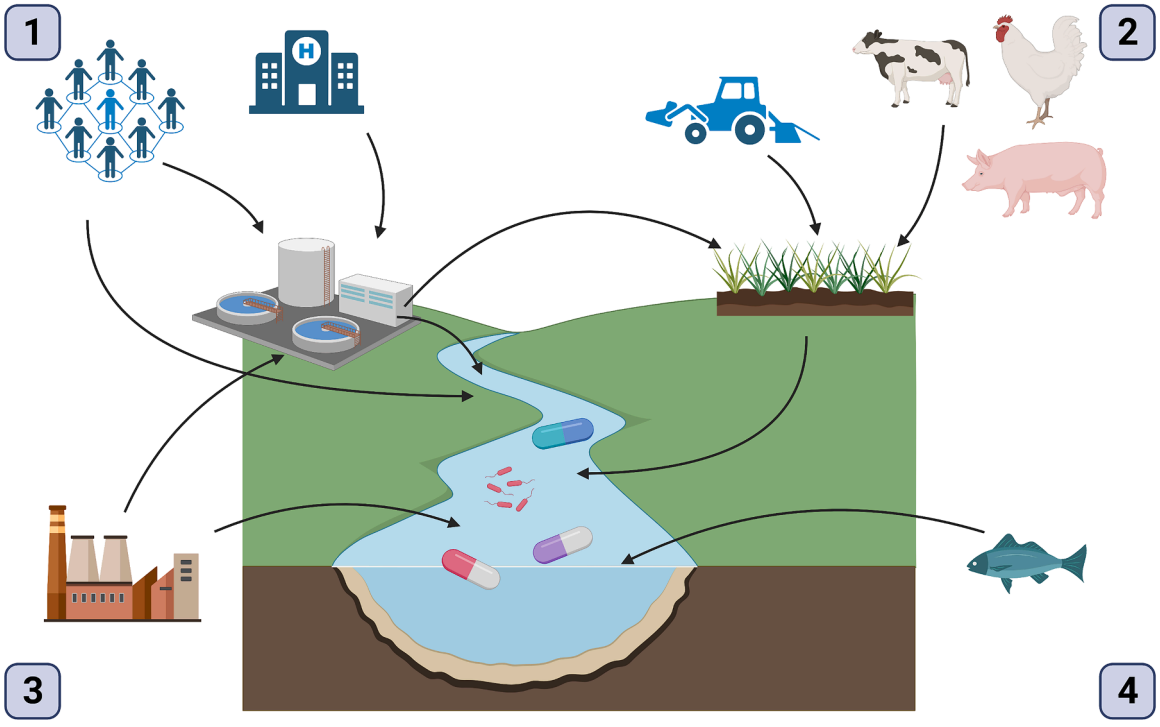

Fig. 1. Potential pathways for antimicrobials (such as antibiotics) to enter the environment. 1. Use in hospitals and the community results in antimicrobials entering the wastewater treatment system. As a result, the compounds and associated metabolites may enter the environment through discharge of wastewater to the water environment or biosolid application to land (which may enter water courses or groundwater through run-off). 2. Use of antimicrobials as plant protection products and in livestock production can result in direct application to agricultural soils, or indirect application through animal manure, respectively. Following rainfall, this can run off into rivers and streams. 3. Antimicrobial production facilities can release antimicrobials into the environment. 4. Aquaculture can result in direct application of antimicrobials or leaching into surrounding aquatic environments. There are other potential sources not included in the diagram, for example leachate from landfill. (Created with Biorender).

Table 1
Definition of terms used in this study and the broader research literature.

Terminology	Definition
Assessment Factors (AFs)	Also known as 'safety' or 'uncertainty' factor. A numerical value used in the derivation of a PNEC. The size of the assessment factor is determined by the uncertainty associated with the available effects dataset with larger assessment factors generally applied when there are less data available.
MIC-based	Applying a combination of eco-evolutionary theory and different mathematical approaches to derive a selective concentration using existing datasets, e.g., MIC databases.
Experimental	Empirical, controlled experiments where variables are manipulated, and changes in resistance endpoints directly measured with a variety of phenotypic and/or genotypic approaches.
Minimal Selective Concentration (MSC)	Determined by calculating selection coefficients for a resistance determinant and plotting these against antimicrobial concentration. At the MSC, resistance should not increase nor decrease over time (Gullberg et al., 2011).
Minimum Inhibitory Concentration (MIC)	The lowest concentration of an antimicrobial where growth of the target organism is completely inhibited.
Lowest Observable Effect Concentration (LOEC)	The lowest tested concentration in an experimental assay, where a statistically significant effect (e.g., on growth, survival, etc.) is observed.
No Observed Effect Concentration (NOEC)	The highest concentration tested in an experimental assay where no statistically significant effect was observed, i.e., the tested concentration directly below the LOEC.
Predicted No Effect Concentration (PNEC)	Concentration of a chemical below which an unacceptable effect on the endpoint of interest, e.g., aquatic ecosystem, is *not* likely to occur, during either short- or long-term exposure. In ecotoxicology, it is derived from available ecotoxicity data, e.g., NOECs and EC10s for chronic effects and EC50 concentrations for acute effects.
Predicted No Effect Concentration for Resistance (PNECR)	Like ecotoxicological PNECs but used to specifically refer to endpoints relating to AMR.

using 10, as it follows recommendations by the European Medicines Agency (EMA, 2018), which may or may not be appropriate for AMR selection as this value was proposed in relation to ecotoxicity data (Murray et al., 2021).

It is important to understand if data used to derive PNECRs are based on experimental resistance endpoints or are generated from MICs. This is discussed in much greater detail in the section 'Interpreting data', but we define experimental PNECRs as using data collected during a controlled experimental approach, where changes in resistance determinants are tracked at different antimicrobial concentrations. Conversely, MIC-based PNECRs will be used to refer to PNECRs that use MICs to predict the selective concentration, rather than directly determining it experimentally.

1.3. Selection for AMR

Even very low concentrations of antibiotics (ng/L) can select for resistant strains of bacteria, relative to isogenic susceptible strains (Gullberg et al., 2014, 2011) and in experiments using complex communities of bacteria (Kraupner et al., 2018, 2020; Lundstrom et al., 2016; Murray et al., 2020; Stanton et al., 2020). These studies and others have raised concerns that contamination of the environment with sub-MIC concentrations of antibiotics (Gullberg et al., 2011) could increase the rate of the evolution, emergence, mobilisation and/or maintenance of AMR.

Several key concepts are important for understanding the methods used in previous studies and their interpretation; these are outlined here. These relate to whether different approaches determine positive selection (an increase in the number of resistant organisms/genes) or increased persistence (reduction in rate of decrease) of resistance, or whether this cannot be confirmed with the approach taken. Both positive selection and increased persistence depend on the relative fitness cost of a resistance mechanism within its genetic, host, community, and/or ecological context.

Generally, AMR is associated with a fitness cost (Andersson and Hughes, 2010), so a resistant cell would be outcompeted by a susceptible cell in the absence of a selective pressure (Fig. 2, 'Absence of antimicrobial'). Selective pressures, in this case, would be concentrations of antimicrobials that negatively impact susceptible cells, thereby ameliorating the fitness cost of resistance. Although AMR generally does confer a fitness cost, there are instances where AMR has no observable fitness cost. These AMR mechanisms are therefore likely to be maintained over time, not lost, even in the absence of selective pressure (Andersson and Hughes, 2012). When no apparent fitness cost is associated with resistance, this can often be attributed to compensatory mutations, which are genomic modifications that allow for the cell's fitness to be maintained despite retention of the resistance genotype (Andersson and Hughes, 2011). Similarly, some AMR is associated with a fitness benefit even in the absence of antimicrobial selective pressures (e.g., Michon et al. (2011)), which causes practical difficulties in determining the MSC, limiting its utility as an approach as resistance determinants may confer a fitness benefit, even in the absence of antimicrobials (Kraupner et al., 2020; Stanton et al., 2020). The MSC itself (Fig. 2, "Maintenance of AMR") is where resistant and susceptible cells have equal fitness, i.e., resistance is maintained over time at the same prevalence unless the MSC is exceeded, or there are stochastic events that alter this balance in fitness (such as changes in environment, compensatory mutations, etc.).

Positive selection results in increased AMR prevalence over time, usually due to the presence of one or more selective pressures (i.e., AMR increases over time when concentrations of antimicrobial agents exceed the MSC, Fig. 2, 'Positive selection for AMR'). Generally, this is thought to occur in a dose-dependent manner, where the selective pressure increases with antimicrobial concentration. However, this is not always the case; one study demonstrated that the magnitude of selection was similar at high, clinically- and low, environmentally relevant antibiotic concentrations (Murray et al., 2018).

Finally, we use 'persistence of resistance' (Fig. 2, 'Persistence of AMR') to indicate a middle ground between loss of AMR in the absence of selective pressure, and the positive selection of AMR in the presence of a sufficiently strong selective pressure. Persistence occurs below the MSC, and AMR is predicted to be lost over time. However, the rate at which AMR is lost is reduced relative to the absence of any antimicrobial, due to the presence of a low-level selective pressure that is not sufficiently strong to result in positive selection (Fig. 2). In other words, the fitness cost of being resistant is only partially offset, so there is still more AMR present at any given time point before AMR is completely lost, than there would be if there were no antimicrobial present (Murray et al., 2018; Stanton et al., 2020). We have identified, where possible, which datapoints reflect positive selection or persistence from the literature search, for the reasons detailed below.

It is important to differentiate between persistence of, and positive selection for, AMR, as their outcomes have different implications for the understanding of risk. With positive selection, there will be cumulatively more AMR (higher prevalence) in the environment over time. Persistence of AMR has implications for exposure, as even concentrations of antimicrobials below the MSC may result in higher levels of AMR than in settings where no antimicrobials are present. Even though prevalence decreases over time with increased persistence, this provides greater opportunities for exposure and subsequent colonisation and/or infection by AMR organisms in the exposed humans, animals or plants (Stanton

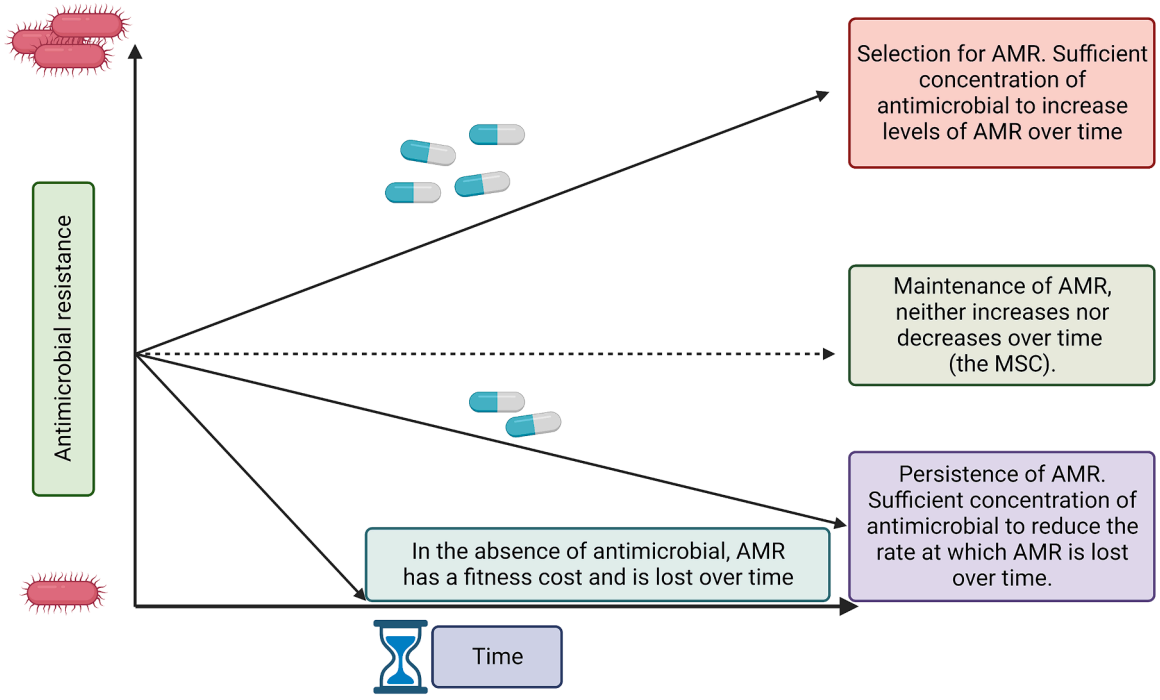

Fig. 2. The possible outcomes in terms of levels of AMR over time (loss/extinction, persistence, maintenance, and selection), for varying concentrations of antimicrobials (i.e., strength of selective pressure). Created with Biorender.

et al., 2020).

Environments that are chronically exposed to concentrations of antimicrobials that do not result in positive selection of AMR, but result in persistence, means AMR could be maintained in that environment for longer than in a pristine environment. Then, combined with intermittent spikes in antimicrobial concentration (exceeding the MSC) resulting from infrequent pollution events (such as those caused by heavy rainfall e.g., runoff from agricultural land/combined sewer overflows), could theoretically result in positive selection of AMR. Cycling of concentrations that result in positive selection and persistence could therefore, hypothetically, result in maintenance of AMR in the exposed environment indefinitely. This would depend on rate of AMR loss, gap between pollution events and concentration (selective strength), amongst other factors. Concentrations that initially cause positive selection will also decrease over time due to degradation or changes in bioavailability but still result in persistence of AMR, resulting in prolonged maintenance of AMR that could be positively selected for in future pollution events.

1.4. Aims of this study

This study aims to provide a summary of selective concentration data for antimicrobials and interpret them in the context of current scientific understanding, to provide recommendations for the establishment of thresholds for antimicrobials in terms of their potential to select for AMR. Advantages and disadvantages of different methods will also be discussed, and recommendations for future research will be provided.

2. Materials and methods

2.1. Generating the database - literature search

To obtain an understanding of the current available selective endpoint data and the approaches used to determine them, a semi-systematic literature search was conducted using the PubMed database. Search terms were designed and tested to ensure they captured known relevant publications ($n = 12$). Following this, search terms were refined, and included terms such as, "MSC" or "minimal selective concentration", AND "antimicrobial" OR "antibiotic" OR "antifungal" or "biocide", AND "AMR" OR "antimicrobial resistan*". The final search terms and the list of key known publications used to verify these can be found in Supplementary File 1. The titles and abstracts for papers identified using these search terms were downloaded and screened for relevance (Supplementary File 2). Inclusion/exclusion criteria are in Supplementary File 3 – Table 1.

2.2. Terms and definitions

A variety of terms are used to describe selective endpoints across studies (Table 1). Note, unlike the MSC, PNECRs generated using NOECs can represent both persistence and selection and thus were recorded either as confirmed 'selection', 'persistence' or whether this was 'unknown' in the curated database.

2.3. Database curation

After title and abstract screening, full texts were downloaded and screened based on the same criteria as above (Supplementary File 2 - "Full text screening", Supplementary File 3 - Table 1). In addition, data were excluded at full text if data were reported inaccurately or unclearly (e.g., inconsistencies in reported values in different sections of the paper). Papers that only included antimicrobial combinations (e.g., antibiotic-adjuvant), or mixtures of antimicrobials were excluded, as were individual datapoints that were for antimicrobial combinations (e.g., antibiotic-adjuvant) or mixtures. LOECs were only recorded if reported in experimental studies where a NOEC was also defined.

Data on published selective concentrations and the methods used to derive them were input into a database (Supplementary File 4, "Database"). Data extracted included: antimicrobial type (e.g., metal, antibiotic, antifungal etc.); antimicrobial class (e.g., tetracycline); individual antimicrobial (e.g., oxytetracycline); MSC, LOEC, NOEC endpoint values reported and their respective units, as well as any reported PNECs, their units and associated AFs; whether selection or persistence was measured or whether this cannot be known from the data reported; the genotype (e.g., gene or mutation, where known); the

genetic context (e.g., chromosomal, or plasmid-borne, if known); the phenotype measured (e.g., if cells were cultured on antibiotic plates); the inoculum (e.g., the bacterial strain, or matrix the community was derived from); the experimental system (e.g., liquid microcosm) including the temperature and growth media used (if an experimental study); the method used to determine endpoints (e.g., qPCR); the bioinformatic pipelines and version (if any were used); the paper reference; and any notes (e.g., to briefly describe the methodology and or any explanations for the data entries). All MSC, LOEC, NOEC and PNECR data entries were double checked for accuracy at least once by two different authors. For simplicity and to facilitate standardisation of data across approaches, in the database, all 'selective' concentrations (i.e., before AF application) were listed as LOECs (i.e., MSCs, size-adjusted lowest MICs etc. were all listed in the LOEC column).

2.4. Standardisation to generate PNECRs

Some publications reported MSCs, whereas others reported LOECs, NOECs, and/or PNECRs. In addition, different units were used across the collated studies. Therefore, to enable a comparison of the collated data, 'standardised' PNECRs were generated using the following steps:

1. MSC and experimental NOEC data had an AF of 10 applied to generate PNECRs. MIC-based data (listed in the LOEC column) had an AF of 10 applied to generate PNECRs.
This AF is in line with current guidelines for environmental toxicity to different organisms, including microorganisms (EMA, 2018). Previously, we suggested MSCs did not require an AF as this may lead to overestimation of risk (Murray et al., 2021). However, for these meta-analyses, it was important that all data were standardised the same way, so any differences did not simply reflect different AFs.
2. Experimental LOEC data with no NOECs were removed. This decision was made because the lowest tested concentration was therefore the LOEC, with no indication where the NOEC may lie.
3. Where PNECR data were reported in a publication, the AF was noted, and if different to 10, the PNECR was multiplied by the AF used in the publication before being divided by the AF of 10 used in this study.
4. All standardised PNECRs were converted to μg/L.

The standardised PNECRs were double checked for accuracy by a second team member. Standardised PNECRs were compared across approaches and systems, including experimental PNECRs vs MIC-based PNECRs, single species vs community experiments, and PNECRs generated using phenotypic or genotypic data.

2.5. Statistics and visualisation

All statistical tests and data visualisation were performed in R Studio (RStudio, 2015). Data were tested for normality using the Shapiro Wilks test and then Wilcoxon Rank Sum tests were used to compare groups, to reflect the unequal sample sizes and non-normal distribution of data. A significance threshold α of 0.05 was used to report significant results. All figures were constructed with the ggplot2 package (Wickham, 2009).

3. Results & discussion

3.1. Summary

Fig. 3 summarises the number of publications/data sources identified from initial searches, how many remained after screening, and the total number of standardised PNECRs that were generated based on the data collated (n = 331 from 21 publications). Of the 331 standardised PNECRs collected, 319 were for antibiotics. Given the lack of data for other antimicrobials, only antibiotics were studied further. The 319 standardised antibiotic PNECRs were then grouped under different classifications:

Experimental PNECRs were classed as PNECRs that had been derived from any type of experiment where changes in resistance endpoints were measured directly (e.g., increases in resistance genes as determined by qPCR or metagenomic sequencing, or increases in proportion of resistant bacteria determined through plating, etc.). The PNECRs excluded from these analyses either modelled MSCs from growth rate data for

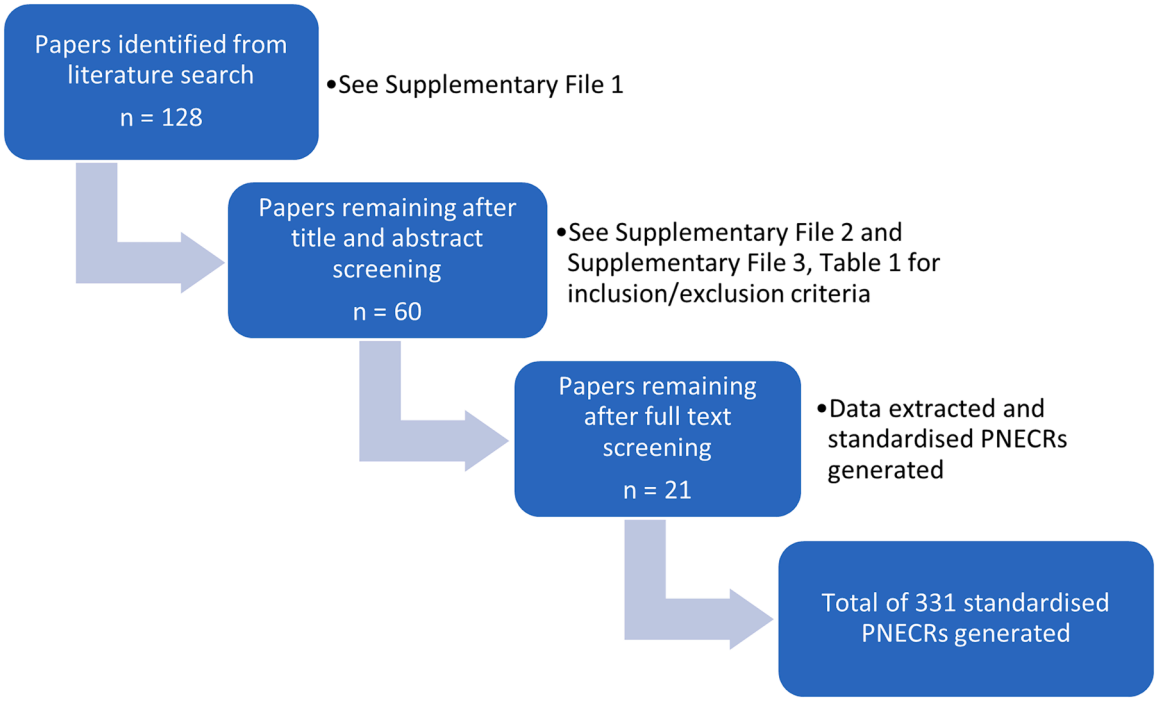

Fig. 3. Flow diagram showing the number of publications/data sources identified by semi-systematic searches, those remaining after title/abstract screening and full text screening, and the total number of standardised PNECR data entries generated from the data located.

individual species (e.g., Frost et al., 2018; Klümper et al., 2019; Vos et al., 2020) or used reduction in overall growth of a community as a proxy for resistance selection (Murray et al., 2020). This is discussed in further detail below.

MIC-based PNECRs were classed as any approach that used a dataset of MICs to estimate PNECRs. Some PNECRs were not covered by either of these definitions and so were excluded from the comparative analyses below (Figs. 5–7). These PNECRs are still recorded in the database, with a note that they were excluded; they are discussed further in the Results & Discussion section.

'Community' or 'single species' experiments, where the experimental inoculum comprised of a community of bacteria (i.e., more than two strains of bacteria), or only two strains of the same species, respectively. For example, the study by Kraupner et al. (2020) had one experimental system where a wastewater community was used to generate a biofilm, and another experimental system used a community of 149 different *Escherichia coli* strains. Both of these were classed as 'community' PNECRs. Conversely, as an example, the study by Gullberg et al. (2011) competed two strains of the same species; these types of experiments were classed as 'single species'.

As a result, of the 319 antibiotic standardised PNECRs, 143 antibiotic PNECRs were classified as 'experimental' and 151 PNECRs were classified as 'MIC-based'; 25 PNECRs were not classified as either. Of the 143 experimental antibiotic PNECRs, 105 were derived from NOECs, with the remaining 38 being MSC-based. Of the 151 antibiotic MIC-based PNECRs, 101 were from a single study i.e., Bengtsson-Palme and Larsson (2016).

3.2. Antimicrobials studied

Antibiotics were the most studied type of antimicrobial, with only 12 of the 331 standardised PNECRs generated belonging to a different antimicrobial class (6 metals, 5 antifungals, and 1 ionophore (Supplementary File 4). Given the limited data for other antimicrobials, we focused on antibiotics for the rest of the study.

Several antibiotics had considerably more standardised PNECRs (Supplementary File 3, Table 2), such as azithromycin ($n = 32$), ciprofloxacin ($n = 24$), clarithromycin ($n = 28$), erythromycin ($n = 25$), tetracycline ($n = 13$) and trimethoprim ($n = 29$). These six antibiotics represent >45 % of all available antibiotic standardised PNECRs reported. In some instances, the majority of these datapoints came from an individual study whereby many different methodologies were trialled, or different endpoints were measured, resulting in a large number of PNECRs (e.g., the three macrolides from Stanton et al. (2020): azithromycin: $n = 28/32$ datapoints; clarithromycin: $n = 25/28$; and erythromycin: $n = 20/25$). In other instances, a variety of studies had produced PNECRs for particular compounds. This was the case for both ciprofloxacin and tetracycline. For ciprofloxacin, PNECR data was generated across nine studies for 24 PNECRs (Bengtsson-Palme and Larsson., 2016: $n = 1$, Gullberg et al., 2011: $n = 4$, Koutsoumanis et al., 2021: $n = 1$, Kraupner et al., 2018: $n = 10$, Murray et al., 2020: $n = 3$, Rico et al., 2017: $n = 1$, Stanton et al., 2020: $n = 2$, Vos et al., 2020: $n = 1$, and Zhang et al., 2022: $n = 1$). For tetracycline, 13 PNECR datapoints were identified and were derived from nine different publications (Bengtsson-Palme and Larsson., 2016: $n = 1$, Gullberg et al., 2011: $n = 1$, Gullberg et al., 2014: $n = 2$, Koutsoumanis et al., 2021: $n = 1$, Lundstrom et al., 2016: $n = 3$, McVicker et al., 2014: $n = 2$, Menz et al., 2019: $n = 1$, Stanton et al., 2020: $n = 1$, and Zhang et al., 2022: $n = 1$). In contrast to antibiotics that were studied in depth by one or multiple studies, one publication generated a single PNECR for over 100 compounds (Bengtsson-Palme and Larsson, 2016). In fact, most antibiotics identified in the dataset only had one standardised PNECR value, which usually arose from the Bengtsson-Palme and Larsson (2016) paper (Supplementary File 4). Antibiotics with a minimum of two standardised PNECRs are shown in Fig. 4. Although there were approximately equal numbers of experimental and MIC-based PNECRs available, for experimental PNECRs, these were skewed towards a handful of antibiotics, limiting the breadth of experimental PNECR data available. These seem to have been biased towards antibiotics deemed 'of concern' such as those listed in the EU Water Framework Directive Watch Lists (Carvalho et al., 2015; Gomez Cortes et al., 2020; Loos et al., 2018).

3.3. PNECR ranges

Standardised antibiotic PNECRs ranged from 0.00087 μg/L (for ciprofloxacin (Koutsoumanis et al., 2021)) to 2000 μg/L (for carbenicillin (Frost et al., 2018)). The 1st percentile of all standardised PNECR data was 0.01 μg/L (rounded to 2 decimal places), meaning that 99 % of all PNECRs collated were greater than 0.01 μg/L. The top six antibiotics with the most standardised PNECRs were azithromycin, clarithromycin, trimethoprim, erythromycin, ciprofloxacin, and tetracycline, all with >10 standardised PNECRs (Fig. 4). Although azithromycin, clarithromycin and erythromycin had a high number of standardised PNECRs available, these were primarily from a single study (Stanton et al., 2020) with total number of studies for these antibiotics being three, four and five, respectively (Supplementary File 4). Conversely ciprofloxacin, trimethoprim, and tetracycline had higher numbers of PNECRs, but these were also from several different studies ($n = 9$, Table 2, Supplementary File 4).

3.4. Overview of approaches used in data sources

We also considered the different approaches used to generate data. A broad variety of approaches were used, these have been discussed according to the variables below.

Culturing conditions: Most used nutrient rich media, which are not environmentally representative, but are standard for most microbial experiments. Interestingly, one study conducted experiments in zebrafish embryos (McVicker et al., 2014), presumably to mimic *in vivo* dynamics. Most experiments used liquid microcosms with different growth media (e.g., Iso-Sensitest or R2 media), with the exception of one study that compared MSCs in liquid and biofilm microcosms and found that MSCs were largely unaffected (Hjort et al., 2022). Most complex community studies used liquid microcosms at high temperatures in rich nutrient media (e.g., (Kraupner et al., 2020; Murray et al., 2020, 2018; Stanton et al., 2020), however, some also used lower temperatures (e.g., Murray et al. (2020)) and/or minimal nutrient media, and established biofilms that were exposed to antibiotics (e.g., Kraupner et al. (2018); Lundstrom et al. (2016)).

Inoculum: Some experimental studies used single species of bacteria (Frost et al., 2018; Gullberg et al., 2014, 2011; Hjort et al., 2022; Klümper et al., 2019; Kraupner et al., 2020; McVicker et al., 2014; Vos et al., 2020; Wang et al., 2022a). The species used most frequently was *E. coli* (Arya et al., 2021; Gullberg et al., 2014, 2011; Hjort et al., 2022;

Table 2
PNECR ranges (all μg/L) for three of the most studied antibiotics. Number of data entries and the number of different publications reporting these values also shown. All PNECR data (i.e., across all approaches) are included. Note, lowest PNECRs reported may represent persistence rather than positive selection for resistance.

Antibiotic	Minimum PNECR	Maximum PNECR	Median PNECR	Number of data points	Number of publications reporting
Ciprofloxacin	0.00087	1.077	0.1	24	9
Tetracycline	0.01	100	1	13	9
Trimethoprim	0.016	6.25	1	29	9

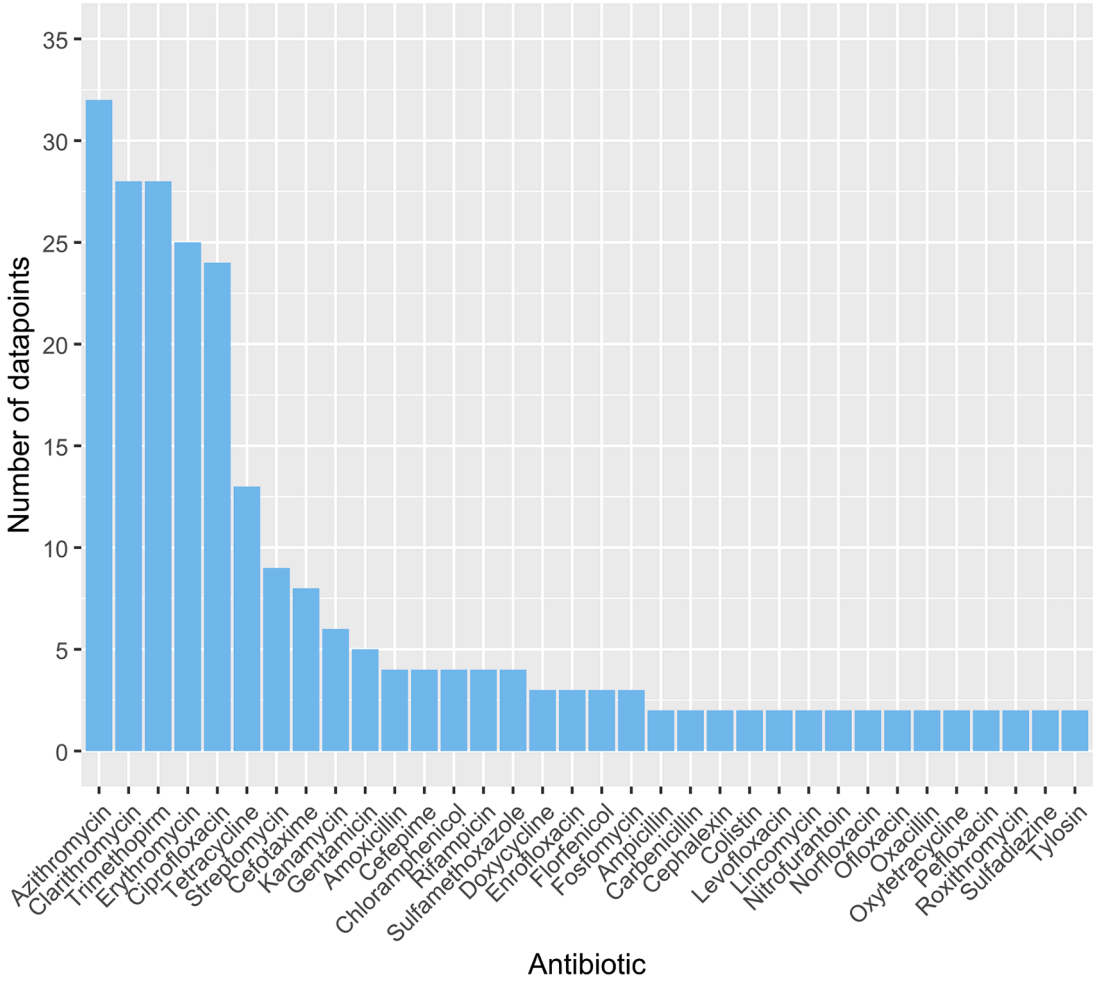

Fig. 4. The total number of standardised PNECRs per antibiotic. Only antibiotics with more than one standardised PNECR are shown.

Klümper et al., 2019; Kraupner et al., 2020; Vos et al., 2020), although other Gram-negative bacteria, such as *Pseudomonas aeruginosa* (Frost et al., 2018), *Comomonas testosteroni* (Wang et al., 2022a) and *Salmonella enterica* (Gullberg et al., 2011) were also used, as well as one study which used the Gram-positive bacterium *Staphylococcus aureus* (McVicker et al., 2014). Individual resistant strains studied harboured a variety of chromosomal mutations, chromosomal resistance genes, or resistance genes carried on plasmids. Some experimental studies used complex communities of bacteria derived from wastewater influent or effluent. These were mostly comprised of different species, but one study investigated resistance in *E. coli* strains that had been collected from wastewater and then evolved under antibiotic exposure (Kraupner et al., 2020).

Analytical method: Selection for resistance in single species assays was measured by tracking increases in the numbers of the resistant strain compared to a susceptible strain, e.g., cell counts via fluorescence-activated cell sorting (e.g. Gullberg et al. (2011)), or colony forming unit counts, via plating (e.g., McVicker et al. (2014)). Sometimes, MSCs were modelled from growth rate data (e.g., Klümper et al. (2019)). Single species were generally not tracked with genotypic/molecular methods. In complex community studies, a variety of different resistance genes and mutations were assessed, using qPCR and/or metagenomics (e.g., Lundstrom et al. (2016); Stanton et al. (2020)), although some also used phenotypic, growth-based methods (e.g., Kraupner et al. (2020); Murray et al. (2020); Stanton et al. (2020)). A variety of different qPCR gene targets and different bioinformatic pipelines were used across these studies.

MIC-based approaches: There were five papers that generated MIC-based PNECRs. As mentioned above, the majority of these PNECRs were from one study (Bengtsson-Palme and Larsson, 2016). This study extrapolated PNECRs from clinical MIC data by taking the size-adjusted lowest 1 % MICs recorded in the EUCAST database for susceptible organisms (i.e., those below the ecological cut-off value), and applying an assessment-like factor of 10 to account for the difference between MIC and MSC.

3.5. *Experimental studies – comparison of standardised PNECRs*

The following section compares standardised PNECRs for antibiotics that we classed as 'experimental', i.e., where changes in resistance endpoints were directly measured to determine a PNECR. Therefore, the following analyses have excluded several PNECRs identified in the main search which did not fit this classification, nor that of MIC-based PNECRs. The PNECRs excluded from these analyses either modelled MSCs from growth rate data for individual species (e.g., Frost et al., 2018; Klümper et al., 2019; Vos et al., 2020) or used reduction in overall growth of a community as a proxy for resistance selection (Murray et al., 2020). Though growth rate has been shown to be one of the most important experimental parameters for determining the MSC (Greenfield et al., 2018) and reduction in community growth has been shown occur at very similar concentrations to selection for resistance marker genes (Murray et al., 2020), changes in resistance endpoints were not directly measured when generating these PNECRs, and so they were not included under the 'experimental' classification.

No *in situ* studies were identified from our search; all experimental studies were conducted under laboratory conditions, mostly *in vitro*. In

experimental studies, one key question is whether assays using single species to study selection for AMR are representative of selection that might occur in the complex communities that exist in the environment. For example, one study showed that the MSC for a focal strain increased in the presence of the community, compared to when the strain was used in a single species competition experiment (Klümper et al., 2019).

We compared all single species and community standardised PNECRs across all antibiotics that had at least one standardised PNECR for each inoculum type (trimethoprim (Gullberg et al., 2014; Hjort et al., 2022; Kraupner et al., 2020; Murray et al., 2020), erythromycin (Gullberg et al., 2014; Stanton et al., 2020), ciprofloxacin (Gullberg et al., 2011; Kraupner 2018; Stanton et al., 2020) and tetracycline (Gullberg et al., 2011, 2014; Lundstrom et al., 2016; McVicker et al., 2014; Stanton et al., 2020), Fig. 5). When comparing inoculum types by each individual antibiotic, there were no significant differences between inoculum types for trimethoprim, ciprofloxacin, and erythromycin. Conversely, for tetracycline, standardised community PNECRs were significantly lower than standardised single species PNECRs (Wilcoxon Rank Sum, $p = 0.019$). However, as noted in the database, all of the single species tetracycline PNECRs ($n = 5$) represented positive selection, whereas none of the community PNECRs ($n = 4$) represented confirmed positive selection, with one of the standardised PNECRs actually being confirmed as increased persistence of resistance. Therefore, the reason the tetracycline community PNECRs were significantly lower than the tetracycline single species PNECRs could be that community PNECRs represent persistence rather than positive selection, though this cannot be known from the data collected. It was not possible to split the overall dataset by persistence or positive selection as there were insufficient data points, with in total, only six data entries recorded as persistence, 83 confirmed as selection, and the remainder ($n = 242$) were classed as 'unknown'.

We also compared single species and community standardised PNECRs across all antibiotics and found, overall, there was no significant difference between the two inoculum types across antibiotics (Wilcoxon Rank Sum, $p = 0.066$). This could be due to the scarcity of data or within-group variation. Overall, there are insufficient data to determine whether single species or community PNECRs are likely to be more protective, but these results suggest it could be compound specific.

Within the experimental data, phenotypic (i.e., culture based) or genotypic (e.g., qPCR, metagenomics) methods were used to measure resistance endpoints. When examining the standardised PNECRs of antibiotic classes and individual antibiotics, most of the antibiotics only had a single standardised PNECR for either approach. Across all antibiotics with at least one genotypic and one phenotypic PNECR (i.e., trimethoprim, erythromycin, ciprofloxacin, and tetracycline, Fig. 6), there was a significant difference between the two datasets ($p = 0.01734$, Wilcoxon Rank Sum). Antibiotics with at least two genotypic and two phenotypic PNECRs were tested individually for significant differences. Only tetracycline had significantly higher PNECRs using phenotypic methods ($p = 0.028$, Wilcoxon Rank Sum). There were no significant differences in PNECRs derived using genotypic or phenotypic methods for the remaining antibiotics tested (erythromycin, and ciprofloxacin; note trimethoprim was not tested as there was only a single

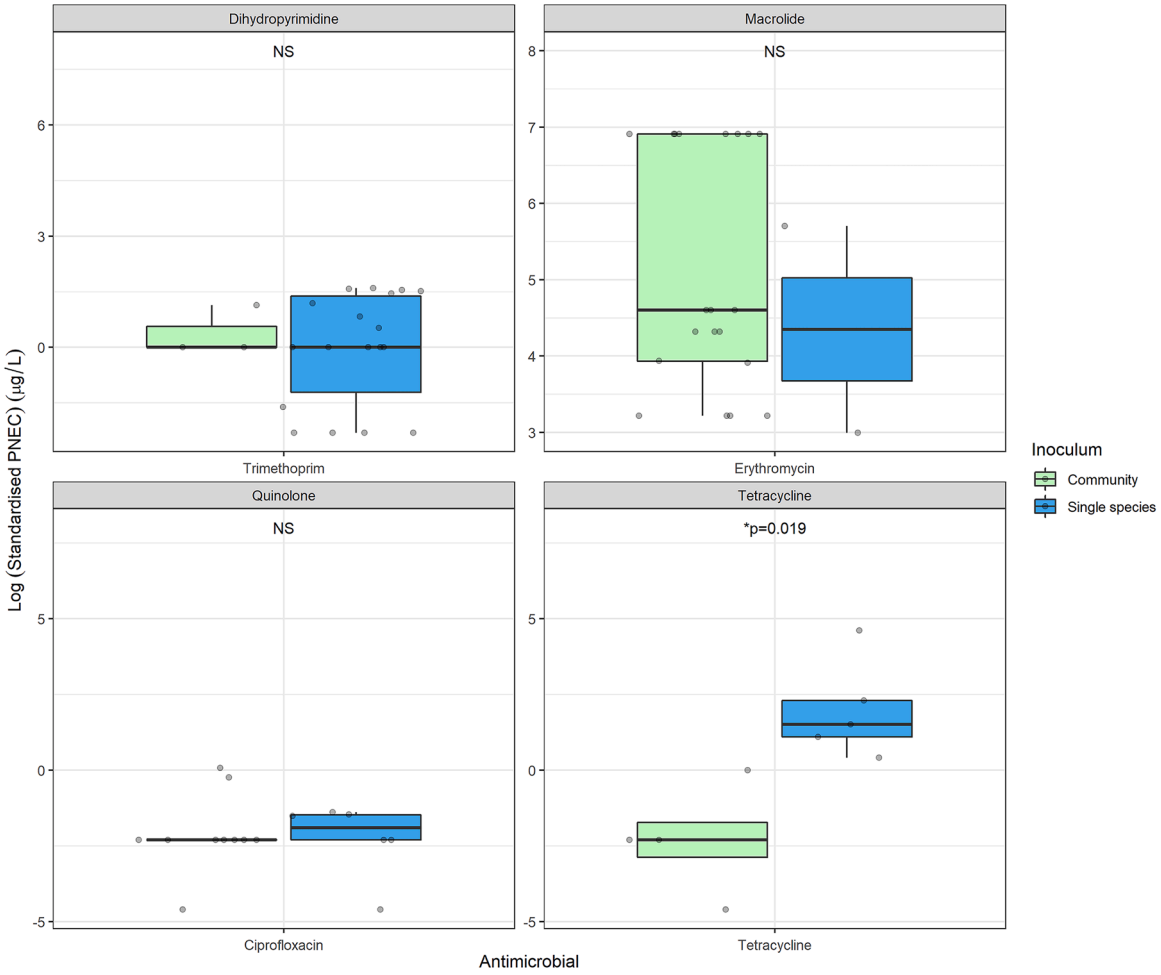

Fig. 5. A comparison of experimentally derived standardised PNECRs (i.e., derived using both MSC and NOEC based approaches) using a single species inoculum or a complex community inoculum. Note, only antibiotics which have both single species and complex community standardised PNECRs available are included. Wilcoxon Rank Sum test used to derive p values. NS = Not significant.

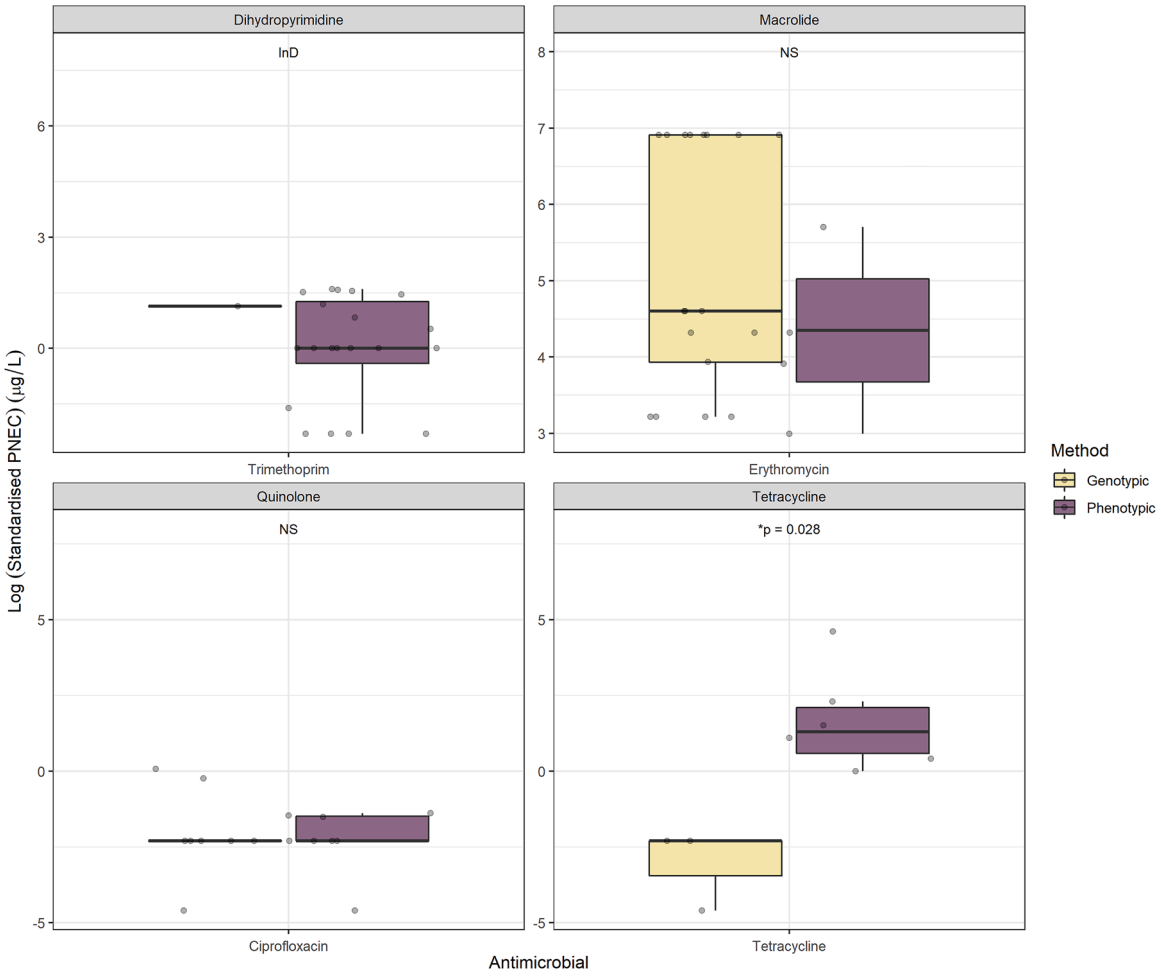

Fig. 6. A comparison of standardised experimental PNECRs by the endpoint type (i.e., genotypic, such as qPCR or sequencing; or phenotypic, such as colony forming units/ml or optical density), split by antimicrobial compound and class. Note, this shows data from experiments using both types of inoculum (i.e., single species and complex community). Only antimicrobials and their respective classes with at least one genotypic and one phenotypic based standardised PNECR are included. Wilcoxon Rank Sum test used to derive p values. NS = Not significant, InD = Insufficient data for statistical testing, as in, only one standardised PNECR available for one of the methods.

genotypic PNECR).

3.6. Experimental vs MIC-based PNECRs

It has previously been suggested that MIC-based PNECRs may be more protective than experimental PNECRs (e.g., Murray et al., 2020). We compared PNECRs for all antibiotic classes and individual antibiotics which had both sufficient MIC-based and experimental data available (i.e., had a minimum of one of each PNECR available, Fig. 7). For statistical testing of individual antibiotics, data were filtered further to only include antibiotics with a minimum of two MIC-based and two experimental PNECRs available (which applied to ciprofloxacin, clarithromycin, erythromycin, rifampicin, streptomycin, tetracycline, and trimethoprim). MIC-based PNECRs were significantly lower than experimental PNECRs across these antibiotics (Wilcoxon Rank Sum, $p < 0.001$). When testing each individual antibiotic, MIC-based PNECRs were significantly lower than experimental PNECRs for trimethoprim ($p = 0.006$), clarithromycin ($p = 0.016$), erythromycin ($p = 0.022$), and ciprofloxacin ($p = 0.003$). MIC-based and experimental PNECRs did not significantly differ for rifampicin, streptomycin, or tetracycline (all $p > 0.05$, all Wilcoxon Rank Sum).

The reason the MIC-based PNECRs were more conservative in several cases may relate to the fact that most experimental studies have used Gram-negative species, or communities dominated by Gram-negative bacteria, whereas MIC-based PNECRs may include MICs for Gram-positive species, including the most susceptible pathogen species in MIC databases (e.g., Bengtsson-Palme and Larsson, 2016). However, all of the antibiotics where MIC-based PNECRs were significantly lower than experimental PNECRs do have some effects on Gram-negative species (i.e., trimethoprim (Gleckman et al., 1981), clarithromycin (Hardy, 1993), erythromycin (Washington and Wilson, 1985), and ciprofloxacin (Campoli-Richards et al., 1988)). It may be that more susceptible organisms (as found in MIC databases) have simply not been tested in the experimental studies. However, this should be balanced against whether these susceptible organisms are likely to be found in the environments where these PNECRs are intended to be used. In addition, acquired resistance in Gram-negative opportunist pathogens is a primary concern, with 9 of the 12 priority pathogens designated by WHO being Gram-negatives, including all three classed as 'critical' priority (WHO, 2017). Experimental systems including Gram-negative organisms give important insights into risk of AMR evolution in these organisms in environmental settings.

3.7. Interpreting data

A key question regarding PNECRs relates to whether MIC-based PNECRs should be adopted considering the emergence of experimental PNECRs, or if they are both suitable but perhaps in different phases of

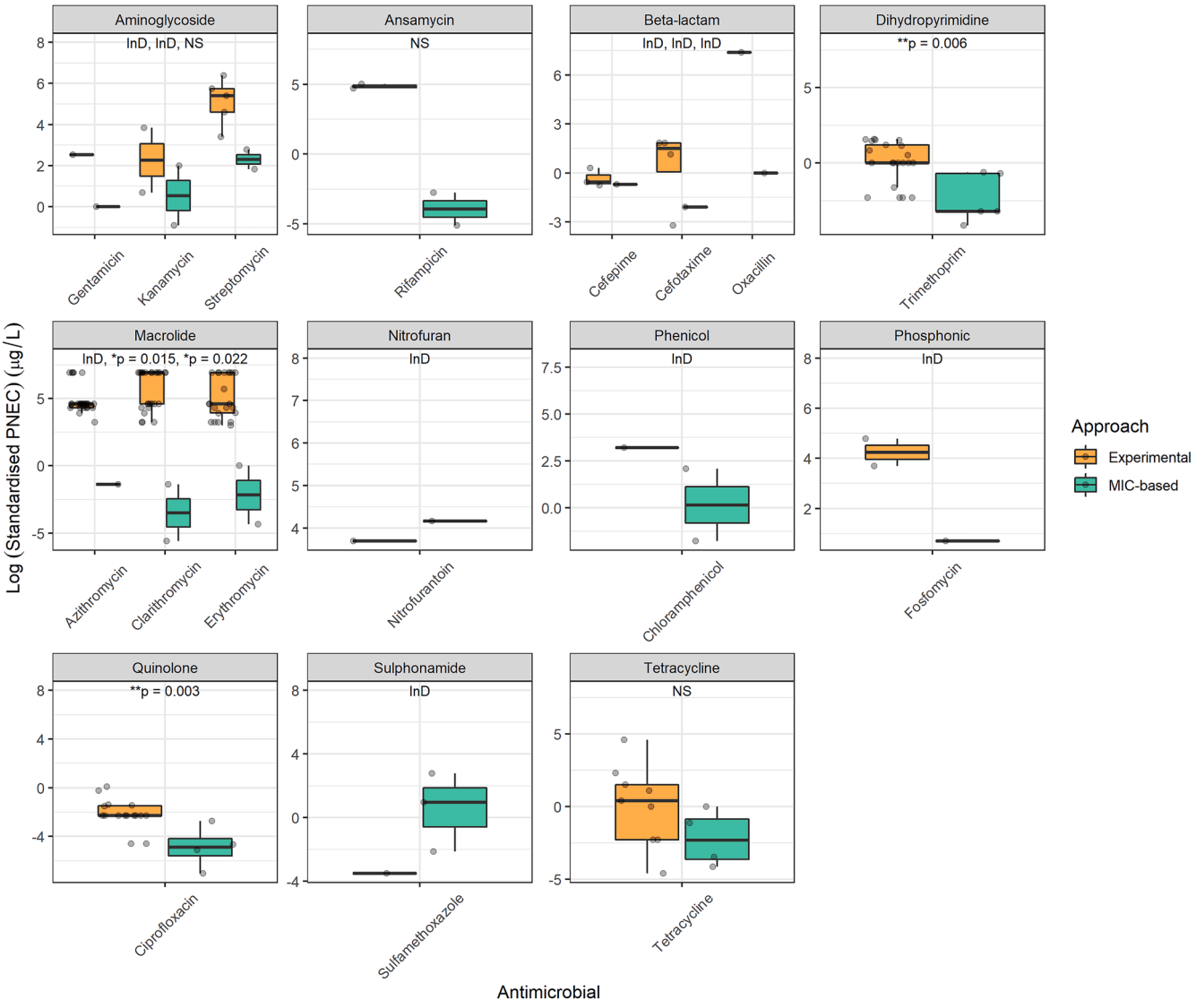

Fig. 7. A comparison of experimentally derived standardised PNECRs (i.e., including single species or complex community inoculum, MSC or NOEC based standardised PNECRs, and all genotypic and phenotypic endpoints) and MIC-based PNECRs. Note, only antibiotics and their respective classes with both experimental and MIC-based PNECR available are shown. Wilcoxon Rank Sum test used to derive p values for antibiotics with a minimum of two standardised PNECRs for each approach (MIC-based or Experimental). NS = Not significant, InD = Insufficient data for statistical testing, as in, only one standardised PNECR available for one or both methods.

assessment. The advantages and disadvantages of each type of approach are discussed below and summarised in Supplementary File 3, Table 3.

The advantages of MIC-based approaches are clear in terms of practicality – they are comparatively cost-effective, rapid, and the use of existing datasets, usually collected according to standardised guidelines, makes comparisons across antibiotics simpler. In the absence of experimental data on selection for resistance, rapid generation of PNECRs for many compounds is appealing from a regulatory standpoint. Studies which adequately report their methods also facilitate testing the reproducibility of the approach, or adaptation of the method as more empirical data emerge. Our analysis of the limited data available also suggests that MIC-based PNECRs appear to be more conservative (at least for trimethoprim, clarithromycin, erythromycin, and ciprofloxacin) and therefore, may offer greater protection against selection for AMR in the environment.

However, there are also several disadvantages to MIC-based approaches, mostly relating to the use of MIC data. The use of MIC data means that selection is not directly measured as standardised MIC collection methods, such as EUCAST (Matuschek et al., 2014), utilise conditions that do not represent the environment (high temperature and nutrient, and unable to capture competition within communities). Furthermore, the data are skewed to clinically relevant strains which may not be present in the environment. To overcome this latter point, some studies have only included data for species that have evidence they can survive in the environment (Tello et al., 2012). A comparison of PNECRs generated using MIC data for clinical vs environmental bacteria would address the extent of this issue; and it is possible that environmental microbes may even be more sensitive than microbes found within EUCAST. MIC-based approaches also tend to apply a single AF value to MIC data to estimate the PNECR. Though AFs may be based on experimental data in some cases (e.g., Koutsoumanis et al., 2021), the relationship between MIC and MSC can vary significantly across different antimicrobial classes and compounds, and even according to the resistance mechanism and its genetic context, e.g., from 4-fold to 230-fold difference (Gullberg et al., 2014, 2011). More experimental data could fine-tune this for MIC-based approaches in future, e.g., according to antimicrobial class or even individual compound. Finally, regarding MIC data, there is a concern that resistance is increasing, and

therefore over time, less protective PNECRs may be generated (which is counterintuitive, as more conservative PNECRs should be adopted as the problem exacerbates) (Murray et al., 2021). This hypothesis could be tested if archived/artificially modified MIC databases were used to estimate PNECRs over time using the same methods.

Furthermore, current MIC-based approaches are unable to capture effects of complex mixtures of antimicrobials that will impact selection in the environment. It is likely empirical studies will be required to adequately inform model parameters to capture mixture effects in the future. Finally, we have already discussed the issue of positive selection vs increased persistence, and there is no way to determine which of these outcomes MIC-based PNECRs might represent.

Experimental studies are advantageous as they are designed to investigate AMR evolution across antibiotic gradients and have the power to reveal complex evolutionary dynamics as well as determining MSCs/PNECRs. Bespoke and flexible experimental design can evolve in line with available technologies/techniques and crucially, in line with current scientific understanding.

The main disadvantages of experimental studies relate to their practicality - experimental PNECRs are more expensive and slower to generate than MIC-based PNECRs. This, alongside the ability to incorporate complexity, for example, using communities (though also an advantage), means that experimental assays may have higher variability and lower reproducibility. However, as no standardisation efforts have been made, there is little understanding of the implications of these two concerns. Like MIC-based PNECRs, experimental studies mostly utilise culturing conditions that may not generate environmentally representative PNECRs. However, this can be modified through experimental design (e.g., Kraupner et al., 2020), and regardless, this could be a fair criticism aimed at many ecotoxicological assays which are already standardised and recommended for use. Both ecotoxicological and AMR assays should be continually improved to be as environmentally representative as possible, with perceived lack of environmental realism being acknowledged but not serving as a barrier to implementation.

Some experimental studies have only utilised single species (e.g., Gullberg et al., 2014; Gullberg et al., 2011), when community context has been shown to influence the MSC (Klümper et al., 2019). However, community experiments are associated with their own specific disadvantages as well, such as population founder effects in daily transfer experiments (though this can be counteracted with pre-enrichment of the culture at the cost of biasing the community), the composition of the community (e.g., predominately Gram-negative communities may be ill-suited to studying antibiotics only active against Gram-positive bacteria) and the depth of data generated available (e.g., in some cases, metagenomics have been used to identify PNECRs for every antibiotic resistance gene that is selected for (e.g. Stanton et al., 2020). This raises a concern of data saturation – there are so many different genes and mutations which could undergo positive selection, making it difficult to define suitable endpoints. One solution may be to define PNECRs based on resistance genes of 'greater concern', e.g., based on human health risk (Zhang et al., 2021). Conversely, richer datasets may lend greater confidence to any PNECRs determined. An important caveat with regards to metagenomics and qPCR is that detection of a given genotype (e.g., presence of a resistance gene) does not necessarily imply a resistance phenotype (e.g., the gene may be present, but not expressed). Using both culture dependent and culture independent approaches moving forward would provide confirmation of phenotype, whilst also providing greater depth and sensitivity of data.

To summarise, both MIC-based and experimental approaches have advantages and disadvantages. Most of the disadvantages associated with MIC-based approaches are inherent to the method, yet MIC-based PNECRs are invaluable when faced with a lack of experimental data. However, MIC-based PNECRs still rely on having (e.g., MIC) data available that have been collected using standardised approaches – and this is lacking for antimicrobials other than antibiotics. Experimental approaches have the potential to be optimised, but this is yet to be fully realised in practice and the breadth of experimental PNECR data available remains comparatively limited. A positive feedback process should be strived for, where MIC-based PNECRs inform experimental data where they are lacking, and then experimental data are used to fine-tune MIC-based PNECRs in the future.

3.8. Knowledge gaps

3.8.1. Antimicrobials other than antibiotics

Our semi-systematic search identified a total of 331 MSC/PNECR data points, 319 of which were for antibiotics. All but one of the 12 metal, antifungal or ionophore data entries were modelled or MIC-based.

Generating effect data and subsequently PNECRs for antifungals is important and should not be overlooked when considering the role of the environment in the selection for AMR. A large proportion of total antifungals used are applied directly to the environment at high concentrations and volumes as plant protection products (Garthwaite et al., 2018). As a result of their use, antifungal resistance has been found in crop pathogens which poses risks to human health, food security and the economy (Fisher et al., 2018). In addition, there is evidence to suggest that clinically-relevant antifungal resistant strains associated with high mortality rates originate in the environment (Rhodes et al., 2022).

As well as minimal amounts of data being available for antifungals and metals, data for entire antimicrobial classes are missing. For example, there is evidence indicating that sub point-of-use concentrations of biocides can co-select for antimicrobial resistance (Murray et al., 2024) yet based on our semi-systematic search, no PNECRs for these exist. Evidence is also emerging that other classes of compounds, for example, non-antibiotic pharmaceuticals (Maier et al., 2018; Wang et al., 2022b, 2023) and other, non-antimicrobial plant protection products (Kurenbach et al., 2015; Liao et al., 2021) may also play a role in AMR selection and dissemination. Therefore, it is likely the range of compounds that will need to be considered for AMR risk assessment in the environment will continue to expand as research progresses (Murray et al., 2024).

3.8.2. Complex mixtures of antimicrobials

There is also limited understanding of how selective/co-selective compounds may interact in complex mixtures. This is an issue relating to assessment of impact of chemicals more generally, not just assessment of the impact on AMR (Backhaus, 2016). In this review, we excluded data for combinations of antibiotics with metals or antibiotic adjuvants, as the data were too few to draw any conclusions. Deriving PNECRs that reflect complex mixture interactions will be challenging given the scarcity of MIC data for antimicrobial compounds such as biocides and metals and uncertainty regarding the nature of interactions whether additive, synergistic or antagonistic. Experimental studies are required to understand mixture effects of antimicrobials, as current mixture modelling approaches are most likely to assume additivity due to lack of experimental data to indicate a different effect (e.g., antagonism) (Rodea-Palomares et al., 2015). However, one study showed that the presence of zinc increased the MSC of ciprofloxacin (Vos et al., 2020), demonstrating that interactions between different antimicrobial classes may be more complex. Environmental context and conditions, alongside the chemical properties of individual compounds, are all likely to influence mixture effects, an important nuance that remains understudied (Murray et al., 2024).

An interim approach could be the application of mixture-specific assessment factors (MAFs). MAFs can be applied when data on the exact concentrations of mixture constituents are unknown (Backhaus, 2016). The MAF is defined by the number of mixture constituents, their respective PNECs, and their proportion in the mixture. For example, the MAF can range from 1 for mixtures which are dominated by a single constituent, to potentially any value, depending on the number of compounds present. However, a recent report suggested that a MAF of

10 was suitable for >70 % of mixtures that were measured in monitoring studies in the aquatic environment (Backhaus, 2021). For mixtures with over 30 constituents, it was also suggested that the MAF could be the number of constituents divided by two (Backhaus, 2021). A recent Environment Agency/UKHSA report also considered this and noted a potential MAF of 5 (EA, 2022). All these recommendations are for use with ecotoxicological data and subsequent risk assessment, but they may also be suitable for risk assessment of AMR. There are no AMR-specific alternative recommendations currently and to the best of the authors' knowledge, MAFs have not been used in the context of AMR.

3.8.3. Applicability to different environmental settings

As discussed above, both estimated and experimental PNECRs tend to be generated using high nutrient, high temperature laboratory conditions. However, where limited studies have compared high temperature, high nutrient experimental systems with lower temperature and/or low nutrient experimental systems for the same antibiotics, PNECRs have not differed substantially, given that current ecotoxicological 'Activated Sludge Respiration Inhibition Test' accepts variability up to 10-fold (OECD, 2009). For example, one study (Murray et al., 2020) used artificial sewage growth media (as per (OECD, 2009)), and reduced the temperature to ambient (21 °C ± 2 °C). Effects of altering these two parameters on PNECR were inconsistent across the four antibiotics tested, but the PNECR reduced by a maximum of 8 test concentrations (16-fold difference) in a single case, with most test iterations not differing at all, or only by a single test concentration ($n = 8$ from total of 12) (Murray et al., 2020). Kraupner et al. (2020) compared liquid microcosms of a community of mixed strains of *E. coli* exposed to antibiotics in high nutrient media and at high temperature, and a more environmentally realistic biofilm derived from sewage effluent maintained in minimal media at room temperature. Both approaches generated the same PNECR (Kraupner et al., 2020). These studies suggest these parameters may not be as crucial as previously thought, however, further data would be needed to confirm this. Experiments could be refined in the future to emulate more environmentally realistic conditions, as well as conducting *in situ* experiments. Though, for the latter, there is a trade-off between environmental realism and cost, time, replicability, variability, and ability to distinguish causation from correlation.

None of the studies captured in this report directly studied MSCs in soil environments, with most experimental study designs being more applicable to aquatic environments (e.g., liquid microcosms). However, some studies have exposed soil communities in experimental plots to antibiotics over prolonged periods and observed increases in antibiotic resistance genes (Brown et al., 2022; Cleary et al., 2016). Though the PNECR data reported here are more applicable to aquatic environments, it would be possible to estimate PNECRs specific to soil and sediment by considering how these chemicals behave in different environmental matrices. Additionally, a study by Elder et al. (2023) presented a framework by which PNECRs for aquatic environments could be used to estimate the risk for selection in the pore water, instead of generating specific PNECRs for soil/sediment. They suggested applying aquatic PNECRs to soil pore water antibiotic concentrations, which were predicted using soil sorption coefficients to calculate the bioavailable fraction of an antibiotic, on the assumption that only the non-absorbed (i.e., within pore water) fraction can select for resistance.

3.8.4. Limitations

In this study we have compared PNECR data across multiple systems, approaches, and publications to guide future research efforts, but it is important to note the limitations of these analyses. Namely, we have not appraised the quality of data/studies, and our searches were not systematic. Further, we have grouped PNECRs together in broad categories and not distinguished between e.g., PNECRs and PNECPs (predicted no effect concentrations for persistence, as suggested previously (Murray et al., 2021)). In part, this reflects the scarcity of data available, although we have still been able to recommend several avenues for future research.

3.9. Recommendations

3.9.1. Standardisation – challenges and opportunities

There are significant obstacles that would need to be overcome to generate a standardised assay (if even a practical long-term goal); decisions need to be made on the following aspects of PNECR determination.

1. *The effect measured.* Metagenomic studies and high-throughput qPCR can both generate large amounts of data without *a priori* decisions on the optimal gene to base the PNECR on. Both are relatively costly, compared to qPCR of single targets, but quantifying single targets risks overestimating PNECRs if the most conservative gene target(s) are not chosen *a priori*. One approach is to use the class 1 integron integrase gene as a measure of selection for genetic platforms (i.e., integrons) that are associated with a wide range of resistance genes (Partridge et al., 2009), this allows a single measure of "gross selection" for AMR to be compared with the same target across antibiotic classes (Murray et al., 2020; Stanton et al., 2020). However, the class 1 integrase gene is unlikely to be the most protective gene target. Even for qPCR assays, there should ideally be some standardisation of reagents, reactions, programmes, and primers across studies, which is yet to be realised, as inter-laboratory variation has been reported to vary by up to 28 % (Rocha et al., 2020). Conversely, phenotypic studies usually quantify prevalence of resistance within a single test species (usually *E. coli*). Given the ability of AMR to be transferred horizontally between bacterial species, focus on a single model organism could underestimate risk. Some ways to identify key gene candidates have been suggested, depending on their relative risk to human health (Martinez et al., 2014; Zhang et al., 2021), but these rankings are yet to be fully developed/adopted. Conversely, model organisms such as *E. coli* can be highly relevant in terms of human exposure and transmission, for example, given it is an opportunistic human and animal pathogen and many standardised approaches exist (Anjum et al., 2021). Studies only using phenotypic data overlook the reservoir of AMR that exists in unculturable bacteria, which may be better represented by culture independent approaches; however, they confirm resistance phenotype.
2. *The relative importance of reproducibility/practicality vs environmental realism.* This applies to both MIC-based and experimental approaches. The worst-case scenario is that PNECRs generated using conditions that poorly mimic the natural environment are not conservative enough. Until sufficient data are generated that confirm this, at best, this risk can be accounted for through application of larger AFs when deriving PNECRs.
3. *Considering complexity.* There are close to infinite possibilities of the resistance mechanisms/genes that can be studied, in different genetic contexts and within different hosts. All of these can impact the PNECR, and it is not feasible to explore all options to find the most conservative approach in each different community/environment. Therefore, decisions need to be made on whether single species, or a standardised microbial community, are required as part of a standardisation process, with the caveat that these are unlikely to generate the most conservative PNECRs. One solution could be to consider multiple endpoints (e.g., a combination of single species phenotypic resistance endpoints and community endpoints to include changes in prevalence of the *intI1* gene and an antibiotic specific resistance gene ranked of high risk to human health, alongside phenotypically confirmed resistance, and effects on community composition) to alleviate the risk that a single endpoint is not sufficiently protective.

4. *The outcome that should be measured.* Is selection or persistence the main outcome of interest? In addition, should threshold concentrations be based solely on where resistant strains are enriched over susceptible strains; or should they also consider the lowest concentration at which resistant strains arise (i.e., where selection for *de novo* mutation occurs)? Most studies examine how resistance that is already established (either through mutation or horizontal gene transfer) may change in prevalence over time. The aspect which has been largely overlooked in studies conducted thus far is emergence of *de novo* resistance, i.e., when novel mutations arise that confer resistance. There is a large body of work on this in a clinical context (exposing clinical pathogens to clinically relevant, or higher, antimicrobial concentrations) to evolve resistant mutant strains. However, there has been very little study of the lowest concentrations of antimicrobials that induce emergence of resistance mutations (Gullberg et al., 2011). We did not include any studies that only determined concentrations at which novel resistance mutations emerge. Most of these define some concentrations which select for novel resistance mutations, without defining the lowest selective concentration. These studies also tend to be conducted in clinically relevant species only. Future research could start to determine the lowest concentrations that select for *de novo* resistance in environmentally and clinically relevant species.

Though lack of a standardisation is in some ways a hinderance, it also offers significant insights. Given all the factors that vary between studies that could impact PNECRs, it is quite remarkable that PNECRs across studies are so similar in some cases (e.g., for ciprofloxacin, Figs. 5 & 6, see also Table 2). Due to the diversity of approaches applied, it can be argued that there should be greater confidence in similar PNECRs generated with different approaches (provided the data are robust), rather than PNECRs generated with a single approach which may not be fully optimised.

3.9.2. Refining existing approaches to further understanding

We identified several opportunities for furthering understanding by using existing approaches with different datasets.

1. *Application of different cut-off values.* Some approaches have taken the 1 % or 5 % MIC values (or HC5 % (5 % Hazardous Concentration) value, in some cases) and used these to extrapolate PNECRs. The reasoning behind these decisions is not always clear, though 5 % has been suggested as the maximum percentage of species within a community that can be affected before ecosystem functioning is compromised (Singer et al., 2011). How relevant this ecotoxicological consideration is to AMR selection is unclear. We suggest that the methods be rerun using these different values, to determine whether they make any material difference to resulting PNECRs. Clearly, if 5 % MIC values are used instead of 1 % MIC values, PNECRs will be higher, but it is not known how significant this difference may be. It would confirm whether a standardised value for these types of approaches should be recommended.
2. *Assessment of relative contribution of Gram-positive and Gram-negative species.* Differences between MIC-based and experimental PNECRs may be, in part, due to the focus on Gram-negative organisms in experimental studies, whereas MIC-based approaches include MIC data for Gram-positive organisms. Existing methods could be rerun using only MICs for Gram-negative bacteria and only MICs for Gram-positive bacteria, to see if PNECRs generated substantially differ. This could inform future experimental studies, which could be optimised for Gram-positives, and the debate around relative risk of resistant Gram-positive and Gram-negative bacteria in the environment. A different AF for each group could then be applied, though how this would be defined is beyond the scope of this study.
3. *Exploration of temporal effects of datasets.* We suggested that one of the issues with MIC-based approaches is that the PNECRs could become less protective overtime. This hypothesis could be rejected if methods were retested using intentionally skewed or archived datasets, methods could then be reconfigured to adjust for rising MICs.
4. *Expansion of MIC datasets.* A long-term recommendation is to generate more MIC data for different antimicrobials, with different usages, against different types of organisms (e.g., environmental species as well as clinical pathogens). This would provide a richer dataset for MIC-based approaches, that could also be compared against current datasets.

3.9.3. Regarding 'definitive' PNECRs

We believe it is not currently feasible to suggest a definitive PNECR for any antimicrobial, given data availability, and the complexity of AMR. However, three of the most studied antibiotics (ciprofloxacin, trimethoprim, and tetracycline) have relatively close agreement across different approaches and different studies. We therefore presented PNECR ranges for these antibiotics (Table 2). Importantly, these ranges should be continually reviewed, evaluated, and adjusted as necessary, particularly as more relevant data emerge.

Another approach could be to set a blanket value for all antibiotics at 0.01 μg/L and then amend this only if evidence emerges this is not sufficiently conservative. If this value were adopted, it means that persistence/selection for AMR should be protected against in 99 % of cases (being the 1st percentile for all PNECRs), based on the available data collated in this study and irrespective of the applied approach or endpoint measured. However, it would not be protective in 100 % of cases, e.g., based on the lowest PNECR for ciprofloxacin reported, which was 0.00087 μg/L. The value of 0.01 μg/L is also in close agreement with the blanket threshold value of 0.05 μg/L derived by the AMR Industry Alliance, based on resistance and ecotoxicological data (Vestel et al., 2021). However, it is an order of magnitude lower than the value proposed by Le Page et al. (2017) where they suggest 0.1 μg/L as a discharge limit for antibiotic manufacturing discharge. This value was suggested to be protective of environmental bacteria, based on traditional ecotoxicological testing, as well as from PNECRs. The PNECRs used to derive this value were taken exclusively from one study (Bengtsson-Palme and Larsson, 2016), which is also included in the database here. When discussing this threshold value, the authors note that the value *"may need to be lower than this for some antibiotics in order to consider the potential to select for resistance in clinical and environmental isolates"* (Le Page et al., 2017).

Finally, it is important to note that the focus of the studies on this topic (that have been used here to derive concentration data) dictates the number of antimicrobials and quantity of datapoints in the database. Therefore, the database is lacking in areas, and in many cases, can be considered 'shallow' in that many antibiotics only have a single PNECR available. As a result of this, we cannot make specific recommendations for other antimicrobials at this time as there are insufficient data.

4. Conclusions

In this study, a database of standardised PNECR data was generated, including metadata regarding the approaches used to calculate them. We identified understudied compounds, as well as compounds for which more data exist so that PNECRs/threshold concentrations can be recommended (ciprofloxacin, trimethoprim and tetracycline). This database can be expanded with a fully systematic search, using multiple literature databases, and added to over time as new data emerge. This database can be used to understand if current MECs of antimicrobials select for AMR, including undertaking risk assessments for specific environments to understand where mitigation strategies may be needed. We hope the data and discussions provided herein will aid policymakers, regulators, and industrial stakeholders in taking further steps to reduce the impact of antimicrobial pollution on selection for AMR, for protection across all One Health sectors.

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CRediT authorship contribution statement

Aimee K. Murray: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Isobel C. Stanton:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Holly J. Tipper:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Helen Wilkinson:** Writing – review & editing, Conceptualization. **Wiebke Schmidt:** Writing – review & editing, Conceptualization. **Alwyn Hart:** Writing – review & editing, Conceptualization. **Andrew C. Singer:** Writing – review & editing, Validation, Investigation, Data curation, Conceptualization. **William H. Gaze:** Writing – review & editing, Validation, Supervision, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Aimee K Murray reports financial support was provided by Environment Agency. Isobel C Stanton reports financial support was provided by Environment Agency. Holly J Tipper reports financial support was provided by Environment Agency. Andrew C Singer reports financial support was provided by Environment Agency. William H Gaze reports financial support was provided by Environment Agency. Aimee K Murray reports a relationship with AstraZeneca that includes: funding grants. William H Gaze reports a relationship with AstraZeneca that includes: funding grants. Aimee K Murray has previously advised the AMR Industry Alliance. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The research data supporting this publication are provided within this paper or are available as supplementary information accompanying this publication.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122310.

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