Determining concentrations of substances that influence development of antimicrobial resistance in the natural environment

Chief Scientist's Group report

October 2024

Project: SC220007/R

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Dr Robert Bradburne
Chief Scientist

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Helen Wilkinson, Ian Martin, Wiebke Schmidt and Alwyn Hart from the Environment Agency scoped and supervised the delivery of the project and contributed intellectually to the final report.

Executive summary

Antimicrobial resistance (AMR) is a significant threat to human and animal health. Though AMR has been traditionally considered a clinical problem, adoption of the One Health approach has resulted in recognition of the role the environment plays in the emergence, evolution, persistence, dissemination, and transmission of AMR.

Evidence suggests that many of these processes may be driven by contamination of the environment with antimicrobials (e.g., Larsson et al., 2018; Larsson & Flach, 2021; Murray et al., 2021), via numerous pathways. One approach to understand the risks posed by antimicrobials in the environment is to determine their potential to increase AMR at measured or predicted environmental concentrations. This approach requires data on 'safe' threshold concentrations, i.e., concentrations which are unlikely to increase levels of AMR in the environment. These data are scarce, with the majority available relating to antibiotics. The limited data is partly due to fact that this is an emerging area of science and the lack of a standardised, widely accepted method to determine the lowest concentration of an antimicrobial that increases AMR. Though a growing body of research in this area exists, several different approaches and experimental systems using different focal species/ communities, analytical tools, and statistical methods have been employed, rendering comparison across studies difficult.

This project collated information on concentrations, reported in the scientific literature, at which selection for resistance may occur on exposure to antimicrobials. These included endpoints such as Minimal Selection Concentrations (MSCs), which is defined as the lowest concentration of an antimicrobial that increases AMR, and PNECRs (Predicted No Effect Concentrations for Resistance). As the endpoints located had been derived using a range of approaches the data was used to derive standardised PNECRs to enable comparison or results arising from different methods.

Consideration of the available data indicated that overall insufficient data was available to draw firm conclusions in relation to comparison of different approaches, but the analysis highlighted that to date the majority of data is available for antibiotics with limited data for other antimicrobials.

The advantages and disadvantages of the different methods are discussed, and recommendations made for future research such as possible improvements to approaches in future, and the need to study a broader range of antimicrobials.

Based on this work, the project team published the peer-reviewed article entitled 'A critical meta-analysis of predicted no effect concentrations for antimicrobial resistance selection in the environment'.

1. Background

1.1 Antimicrobial resistance and the environment

Antimicrobial resistance (AMR) is the ability of microorganisms (bacteria, fungi, viruses, parasites) to resist treatment with, or exposure to, antimicrobial drugs or other antimicrobial compounds, e.g. disinfectants. AMR infections are already 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 100 trillion US Dollars loss in Gross Domestic Product (GDP) (O'Neill, 2016).

The One Health approach to AMR recognises that the health of humans, animals, and the environment are all interconnected (Government, 2019; WHO, 2015). The environment is of concern as it is a reservoir of antimicrobial resistance genes that can contribute to emergence of AMR in human pathogens. In addition, the evolution, persistence, and dissemination of antimicrobial resistance through the environment, and transmission of resistant pathogens to humans via environmental exposure are of concern. Interdisciplinary actions in all relevant sectors, e.g. clinical and agricultural are required to maximise the chances of curbing the emergence, maintenance, and selection (evolution) of AMR, and of reducing the potential for a ‘post-antimicrobial era’. Without effective antimicrobial stewardship, society risks reverting back to the ‘dark ages’ of human medicine, where previously easy-to-treat infections become fatal. In addition, impacts on both food security, through resistance in crop and livestock pathogens, and the global economy are also likely to be severe (O'Neill, 2015, 2016).

The use of antimicrobials in human and veterinary medicine, as well as their use in, for example, personal care products, plant protection products and disinfectants, provides a multitude of pathways by which they can enter the environment (Figure 1). The amounts of antimicrobials reaching the environment depend on how they are manufactured, used, and disposed of and the extent to which they are metabolised in the human/ animal treated. In addition, factors such as chemical degradation, environmental mobility, and the characteristics of the receiving environment such as extent of dilution influence the concentrations detected in the environment.

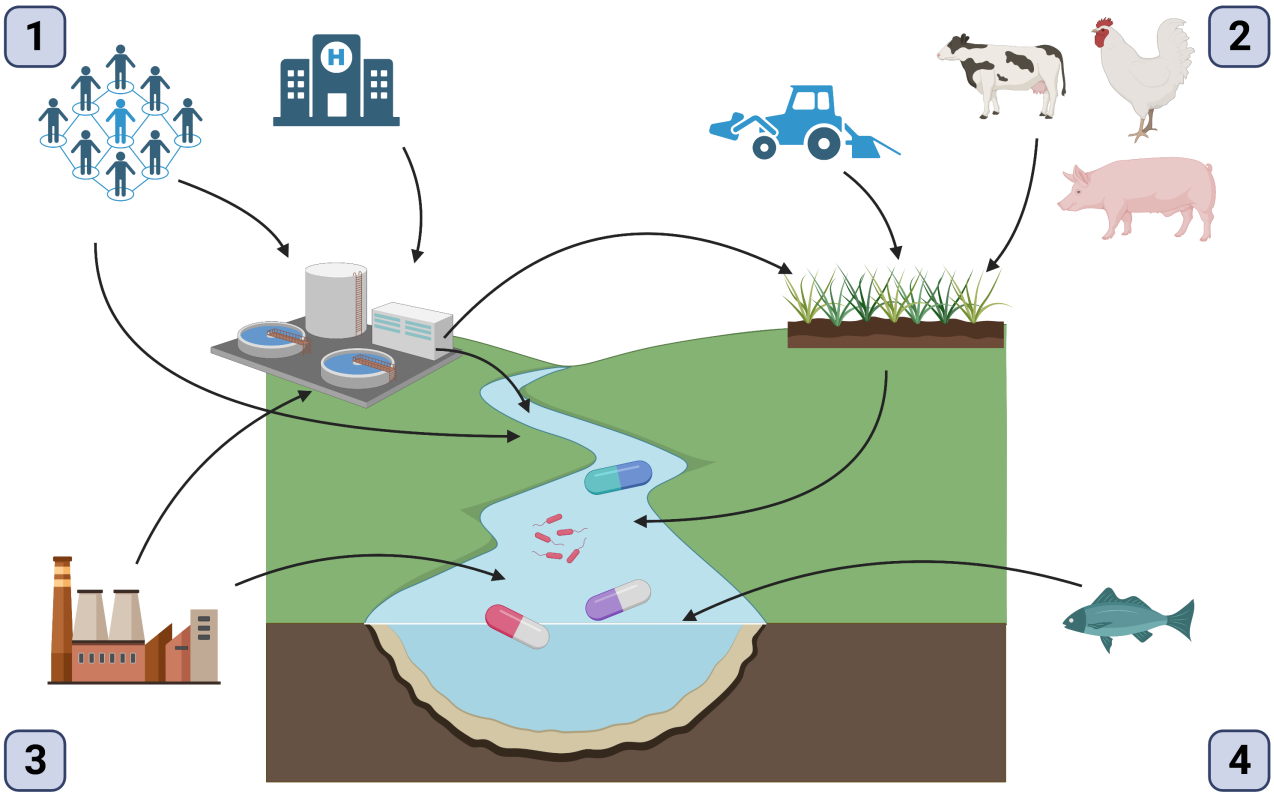

Figure 1. Potential pathways for antimicrobials (such as antibiotics) to enter the environment. 1. Use in hospitals and the community results in antimicrobials entering the wastewater treatment system. As a result, the compounds and associated metabolites may enter the environment through discharge of 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)

As antimicrobial resistant organisms and resistant genes may be present in environments contaminated with antimicrobials this provides opportunities for the selective enrichment of resistant human, animal and/or plant pathogens due to their exposure to antimicrobials in the environment.

In addition, selective pressures posed by exposure to antimicrobials provide opportunities for emergence of resistant microorganisms and/or mobilisation of novel and/or clinically relevant resistant genes from the environment into human, plant, or animal pathogens (Figure 2). AMR can arise through mutations in the chromosomal DNA of microorganisms, and in bacteria, mutations can arise within resistance genes on mobile genetic elements

(such as plasmids, which are extrachromosomal circular pieces of DNA). Through a process called horizontal gene transfer (HGT), plasmids and other mobile genetic elements can be mobilised (shared) across bacterial populations. Where AMR genes are present in these mobile genetic elements this can result in the spread of AMR. Evidence suggests that frequent HGT events only occur in bacteria and archaea (prokaryotes). Acquisition of resistance can occur in the absence of antimicrobials, but presence of antimicrobials can exacerbate/accelerate the process.

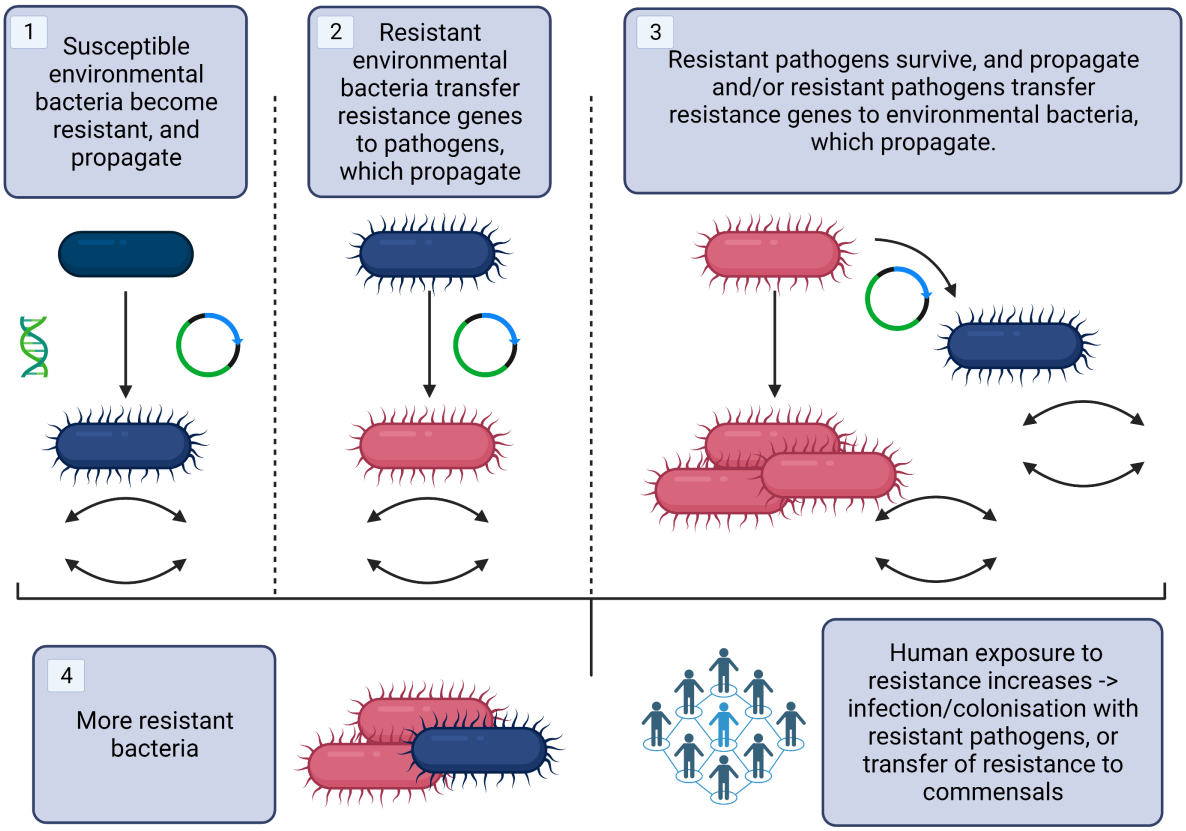

Figure 2. Potential outcomes from exposure of microorganisms to antimicrobials in the environment – using bacteria as an example. The above processes can occur in the absence of antimicrobials, but presence of antimicrobials can exacerbate/ accelerate the processes**.** Bacteria can become resistant (represented by change from blue to red) through mutations in DNA (represented by blue/green DNA double helix), or acquisition of AMR genes through horizontal gene transfer (HGT, example shown with plasmid transfer, plasmids = circular blue/green/black pieces of DNA). 1. Previously susceptible environmental bacteria mutate or acquire resistance genes through HGT and become resistant. 2. Resistance in environmental bacteria is mobilised (i.e., horizontally transferred) to previously susceptible human pathogens, which become resistant. 3. Resistant pathogens of human and/or animal origin can survive and propagate in the environment and could transfer resistance into environmental bacteria, which may be better adapted to survival in the environment, thereby expanding the pool of resistance that could be mobilised to pathogens in the future. All these outcomes result in increased occurrence of resistant organisms/genes (4), which increases the probability of human exposure to resistant

genes/organisms, and subsequent infection with resistant pathogens, colonisation with resistant pathogens, or transfer of resistance from pathogens or non-pathogens into commensal bacteria. (Created with Biorender)

In the clinical environment antimicrobial resistance is determined when microorganisms such as bacteria and fungi no longer respond to antimicrobial medicines which results in the drugs being ineffective. This commonly means that an organism displays growth above a minimum inhibitory concentration (MIC) of an antimicrobial that would kill or stop susceptible organisms growing. However, it has been found that exposure to sub-inhibitory (i.e., sub-MIC) antimicrobial concentrations can still result in enrichment of AMR because resistant organisms can grow faster than susceptible strains that display a reduced growth rate in the presence of antimicrobials. Experimental studies using single species of bacteria have shown that even very low concentrations of antibiotics (ng/L) can increase the number of ('select for') resistant strains of bacteria, relative to isogenic susceptible strains (identical other than differing susceptibility profiles) (Gullberg et al., 2014; Gullberg et al., 2011). Further studies have shown that sub-inhibitory selection by antibiotics can also be observed in experiments using complex communities of bacteria (Kraupner et al., 2018; Kraupner et al., 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-inhibitory concentrations of antibiotics (Gullberg et al., 2011) could increase the rate of the evolution, emergence, mobilisation and/or maintenance of AMR.

As sub-inhibitory antimicrobial concentrations can contribute to the development of AMR, it is important to determine the lowest concentration at which this occurs, to better understand the role of antimicrobial environmental pollution in the enrichment of AMR. Within the research community, different approaches have been used to determine these concentrations, including experimental based methods and those that use existing data such as MIC data.

To understand the current state of the science, this report aims to collate available information on concentrations at which selection for resistance has been reported along with information on the approaches used to determine these concentrations. Further information on the relevant endpoints and the processes that can affect selection for resistance are outlined in Section 1.2 and 1.3, respectively.

1.2 Endpoints used to assess effect concentrations for resistance selection

Several terms referring to selective endpoints/ thresholds/ effect concentrations are used in this report. These have been used somewhat interchangeably in the scientific and regulatory communities, but each have distinct meanings. Within this section, definitions around these terms are provided for clarity to the readers.

1.2.1. The Minimal Selective Concentration (MSC)

The Minimal Selective Concentration (MSC) was first introduced by Gullberg et al (2011). It was initially determined in experimental studies by direct competition between resistant and susceptible strains of the same species (e.g., Gullberg et al., 2014; Gullberg et al., 2011), but more recently experimental studies have also determined MSCs of antibiotics in complex community experiments (e.g., Murray et al., 2018; Stanton et al., 2020). The MSC is determined by calculating selection coefficients for the resistance determinant (y axis) and plotting these against antibiotic concentration (x axis) (Gullberg et al., 2011)). Selection coefficients represent the change in resistance over time, e.g., a positive selection coefficient means resistance will increase in prevalence over time, whereas a negative selection coefficient means resistance prevalence will reduce over time. The MSC itself is the concentration at which the line crosses the x axis (i.e., selection coefficient (y axis) = 0), representing the antibiotic concentration at which resistance will neither increase nor decrease over time in studied species or communities (see Section 1.3.).

1.2.2. Lowest observed effect concentration (LOEC)

LOECs are endpoints usually considered in ecotoxicity tests. In terms of resistance, the LOEC is the lowest concentration tested where a statistically significant change (e.g., increase in number of resistant bacteria, or increase in prevalence of resistance genes) occurred.

1.2.3. No observed effect concentration (NOEC)

NOECs are defined in ecotoxicological experiments as the highest concentration tested where no significant effect on an endpoint (e.g. growth) was observed. In terms of resistance, they represent the highest concentration tested where no statistically significant change is observed (e.g., no significant increase in number of resistant bacteria or no significant increase in prevalence of resistance genes).

1.2.4. Predicted no effect concentrations for resistance (PNECRs)

Some studies have taken MSCs a step further and applied Assessment Factors (AFs) to MSCs, to generate Predicted No Effect Concentrations for Resistance (PNECRs, e.g., (Stanton et al., 2020)). PNECRs however have been determined in a variety of ways. For example, PNECRs have been generated from Minimum Inhibitory Concentration (MIC) data for individual species of bacteria (e.g., Bengtsson-Palme & Larsson, 2016). 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, comprising of many different species (e.g., (Murray et al., 2020)). Such studies tend to use AFs of 10 to reflect the uncertainty in extrapolating from laboratory to field experiments, but use of different AFs to reflect whether the data are from single species or complex communities has not been considered. In experimental studies, AFs are usually applied to NOECs to generate PNECRs.

PNECRs are sometimes are just referred to as Predicted No Effect Concentrations (PNECs, e.g., (Kraupner et al., 2020)). Throughout this report however, we will use the PNECR term, even if the original study reported the value as a PNEC. We recommend using PNECRs in all cases referring to AMR, to distinguish them from ecotoxicological PNECs that are often derived from standardised tests.

There is one thing that all PNECRs have in common – the application of an AF. There has been little discussion around use of different AFs in this context, with most studies using 10, as recommended by the European Medicines Agency (EMA, 2018) based on ecotoxicity data.

One final important point relating to PNECRs is whether the data used to derive them are resistance endpoints that have been directly measured experimentally or if MIC data have been used. This is discussed in much greater detail within the Results section, 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 which have used MICs to predict what the selective concentration might be, rather than measuring it experimentally. MIC data are empirically derived, using standardised methods, but they are conducted primarily for informing clinical practice. The relationship between MIC and MSC is known to vary significantly, by antibiotic and even resistance mechanism or genetic context of that mechanism (Gullberg et al., 2014; Gullberg et al., 2011). Therefore, these MIC-based PNECRs make assumptions about the MIC/MSC relationship without measuring changes in AMR abundance experimentally. There are a further small number of studies using growth rate of bacterial species or communities as a proxy for selection and these are not included in the metanalysis but are discussed in more detail in Section 4.3.

1.3 Key concepts

Several key concepts are important for understanding the methods used to derive the PNECR and MSCs outlined in this report and their interpretation. These mainly relate to whether the approaches discussed are considering endpoints for selection for resistance (an increase in the number of resistant organisms, or resistance genes) or persistence (reduction in rate of decrease) of resistance, or whether this cannot be confirmed with the approach taken. Both selection and persistence depend on the relative fitness cost, or advantage, of a resistance mechanism within its genetic, host, community, and ecological context.

Resistance here refers to acquisition of a mechanism that reduces antimicrobial susceptibility, enabling an organism to outcompete more susceptible organisms on exposure to antimicrobials and so increase in relative numbers. This may also allow the microorganisms to survive antimicrobial treatment whereas a susceptible organism would be killed or would be unable to grow. However, not all resistance mechanisms confer clinical resistance, meaning that some “resistant” organisms that have reduced susceptibility at low antimicrobial concentrations may still be clinically susceptible.

Generally, AMR is associated with a fitness cost (Andersson & Hughes, 2010). This means that a resistant bacterium would usually be outcompeted by a susceptible strain of the same species. This is because for most organisms the expression of the resistance mechanism confers an additional metabolic burden. For example, a resistance mechanism that produces more of an enzyme which degrades antibiotics, such as the production of beta-lactamases, which break down beta-lactam antibiotics, would mean the host cell requires more resources to produce these enzymes. By redirecting these resources toward enzyme production, this would affect the growth rate of the resistant cell, thereby allowing susceptible cells to reproduce more rapidly, and therefore eventually outcompete the resistant cell to extinction (Andersson & Hughes, 2012). Essentially, the fitness cost of acquiring and maintaining a given resistance mechanism usually means that in the absence of a selective pressure, that resistance mechanism will be lost over time (Figure 3).

Selective pressures in this case would be concentrations of antimicrobials at a level that would start to negatively impact the growth of susceptible cells, thereby ameliorating the fitness cost of being resistant. In other words, this is the minimal selective concentration (MSC), as first defined by Gullberg et al. (2011), "…where the fitness cost of the resistance is balanced by the antibiotic-conferred selection for the resistant mutant". This is where the fitness cost of resistance is completely offset, but the resistant cell is not yet benefiting from a fitness advantage that would allow it to grow more rapidly than a susceptible cell. In other words, at the MSC, neither the resistant nor susceptible strain outcompete each other, their proportions in the community remain stable over time. However, an important caveat is that 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 & Hughes, 2012). Further, compensatory mutations can occur, which overtime reduce the fitness cost of AMR (Andersson & Hughes, 2011). Similarly, some AMR is associated with a fitness benefit (e.g., (Michon et al., 2011)), which causes practical difficulties in determining the MSC. This is because in some cases, the growth rate of resistant strains can be faster than susceptible strains, even in the absence of antimicrobials. In this instance, the effect concentration (e.g., lowest observed effect concentration (LOEC)) is determined as the lowest concentration where a relative increase in relative abundance of AMR is observed compared to a no antibiotic control (Murray et al., 2021; Stanton et al., 2020).

Selection for resistance results in increased AMR prevalence over time, usually due to the presence of selective pressure (i.e., AMR increases over time at concentrations above the MSC, Figure 3). Generally, this is believed to occur in a dose-dependent manner, where the selective pressure increases with antimicrobial concentration. However, this may not always be the case, with one study (Murray et al., 2018) demonstrating that the magnitude of selection was largely equivalent at clinically relevant antibiotic concentrations as at environmentally relevant antibiotic concentrations. Murray et al. (2018) observed that the selective pressure (and therefore abundance of AMR) only increased in a dose-dependent manner at the lower, environmentally relevant concentrations tested, before plateauing at the mid-high (clinically relevant) concentrations. Significant effects on survivability were observed at the very highest concentrations tested (Murray et al., 2018).

Finally, we use 'persistence of resistance' to indicate a middle ground between reduction in the prevalence of resistant organisms or genes in the absence of selective pressure, and an increase in the presence of selective pressure above the MSC. Persistence occurs below the MSC, at concentrations where AMR will still be lost over time. However, the rate at which AMR is lost is reduced, due to the presence of low-level selection (Figure 3). 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 extinction than there would be if there were no antimicrobial present (Murray et al., 2018; Stanton et al., 2020). The significance of this is discussed below.

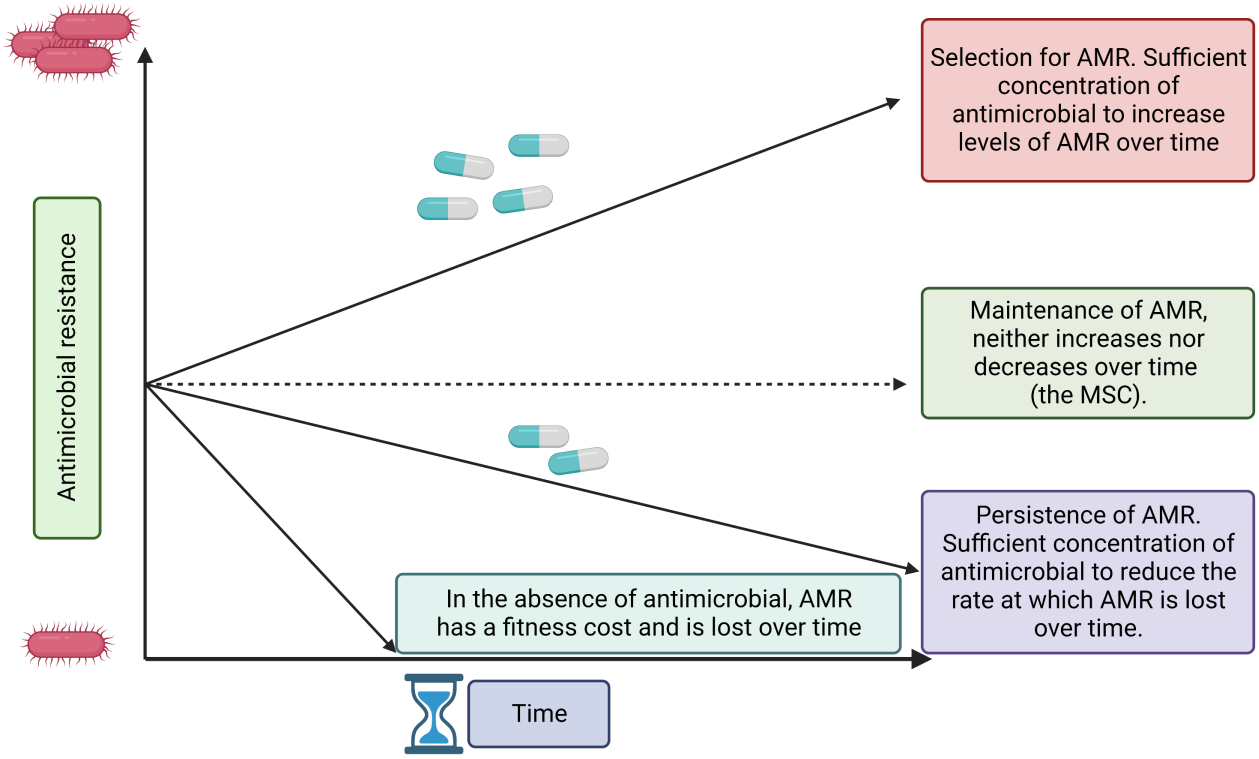

Figure 3. Overview of possible outcomes in terms of levels of AMR over time (loss/extinction, persistence, maintenance, and selection), for varying concentrations of antimicrobials (i.e., strength of selective pressure).

It is important to differentiate between persistence and selection, as their different outcomes have different implications for assessment and understanding of risks posed by AMR in the environment. The obvious risk of selection is that there are cumulatively more resistant micro-organisms/genes in the environment over time. AMR persistence also has implications for potential human and/or animal exposure to AMR in the environment, as even concentrations of antimicrobials below the MSC would result in levels of AMR remaining at higher levels of AMR for longer than in pristine environments (where no antimicrobial agents are present), providing greater opportunities for exposure and subsequent colonisation and/or infection by AMR organisms in the exposed humans or animals (Stanton et al., 2020).

As well as levels of antimicrobial contamination, the frequency and length of antimicrobial contamination also comes into play when interpreting the relative risk of AMR, particularly when considering persistence. Hypothetically, if environments are chronically exposed to concentrations of antimicrobials that are not selective, but do result in persistence of AMR, this means AMR would be maintained in that environment for much longer than in a pristine environment. Then, intermittent spikes in antimicrobial concentrations above the MSC resulting from infrequent pollution events (such as those caused by heavy rainfall e.g., runoff from agricultural land/combined sewer overflows) could positively select for AMR. Cycling of concentrations that result in positive selection and persistence could therefore, hypothetically, result in maintenance of AMR in the exposed environment indefinitely. Further, selective concentrations may decrease over time due to degradation or changes in bioavailability due to sorption. As a result, they may become persistence inducing concentrations, resulting in longer maintenance of AMR in the environment. These are all important factors to consider, given antimicrobial polluted environments can also be polluted with resistant organisms (e.g., wastewater receiving environments, and runoff from sewage/animal manure amended agricultural land).

In a previous study (Murray et al, 2021), it was suggested that persistence of resistance should be the endpoint used in human health risk assessments, in environments where humans are likely to be exposed to resistant pathogens, whereas the selection for resistance could be better suited to environmental risk assessment generally. This is due to the aforementioned increases in opportunities for human/animal exposure that would arise from persistence, necessitating a more conservative approach. However, any increase in resistance compared to an experimental control has been suggested as the endpoint that should be used, regardless of whether this represents persistence of, or selection for, AMR (Kraupner et al., 2020). This is because persistence and selection are highly dependent on factors mentioned above that influence the fitness cost of AMR (the mechanism itself, and its genetic, host, community, and ecological context).

Most interest in the research community and from policy makers has to date focused on MSCs. The MSC term has been used even when describing PNECRs, even though MSCs can only represent selection whereas PNECRs may be used to describe positive selection or persistence. In this study it has been indicated whether the endpoint measured was selection or persistence, or whether this cannot be determined from the data reported. This may be useful for future discussion regarding suitable endpoints to consider when developing suitable thresholds.

Finally, all the above describes whether resistant strains increase, decrease, or remain stable compared to susceptible strains within the same population, i.e., how resistance that is already established (either through mutation or HGT) may change in prevalence over time. The aspect which has been 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 involving 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). Selection for *de novo* mutations

at environmentally relevant concentrations of antimicrobials should be considered in future research efforts.

1.4 Context

As mentioned above, to understand if the presence of antimicrobials in the environment can result in increased AMR, it is necessary to compare the measured or predicted environmental concentrations (MECs or PECs, respectively) with a threshold for selection for resistance, i.e., a PNECR. Where the environmental concentration is greater than the PNEC there is the potential for selection to occur (e.g., Haenni et al., 2022; Hayes et al., 2022; Murray et al., 2021; Murray et al., 2020; Stanton et al., 2020.). The availability of both types of data (i.e., environmental concentration and selective threshold concentration) are lacking for the majority of antimicrobials other than antibiotics.

In terms of environmental concentrations, some monitoring has been undertaken in the UK. For example, monitoring to determine concentrations of several antimicrobials in wastewater influent, effluent and untreated sludge and biosolids was undertaken through the Chemicals Investigation Programme (CIP2 and CIP3), funded by UK Water Industry Research (UKWIR) (UKWIR, 2020, 2022). In the European Union, the Water Framework Directive (WFD) 'Watch List' has included antimicrobials (Carvalho et al., 2015; Gomez Cortes et al., 2020; Loos et al., 2018). Inclusion of a substance on the Watch List requires EU member states to monitor for these substances for a set period. AMR endpoints for selection for resistance, e.g., PNECRs have been considered during the prioritisation process for identifying substances for inclusion on more recent versions of the Watch List (Gomez Cortes et al., 2020).

Environmental risk assessments for antimicrobials currently use PNECs based on ecotoxicity tests that consider endpoints such as growth, survival, etc. in aquatic organisms such as fish, invertebrates, and algae. As AMR endpoints are currently not considered, and selection for resistance has been found to occur at concentrations below reported effect concentrations for microorganisms, i.e. the Minimum Inhibitory Concentration, there are concerns that PNECs based on ecotoxicology data may not be low enough to protect against selection for AMR (Kraupner et al., 2020; Murray et al., 2020). There has been limited progress towards a standardised method that can determine selective threshold concentrations for both new antimicrobials coming to market and those already in use. Though selective endpoint data are being generated, many different experimental approaches are used. This includes using different bacterial species or bacterial communities; in different experimental systems (e.g., biofilm or planktonic); where the effect endpoint being measured differs (e.g., increases in phenotypic or genotypic resistance prevalence). Even the methodology for measuring the same endpoint can differ between studies (e.g., the same gene target, but with different qPCR primers, or metagenome studies using different bioinformatic pipelines to assign resistance genes). Further, an increasing number of approaches generate PNECRs from MIC data, using different sets of MIC data, and extrapolating PNECRs from them in different ways.

A clear summary and evaluation of these different approaches is needed so that future (research) efforts can be guided to produce useful data. This would also facilitate discussions around the basis of PNECR data generated thus far to inform future use of these values in terms of the assessment of selection for resistance.

Most of this report will focus on antibiotics, as the available PNECR data are almost entirely skewed towards this antimicrobial class. However, it is important to note that PNECR data for other antimicrobials will need to be considered as they become available.

2. Project aims

The aims of this project were to:

- Collect available MSC and PNECR data for antimicrobial agents, for microorganisms, derived from both experimental and MIC based approaches.
- Critically evaluate ‘experimental’ and ‘MIC based’ data and set out the strengths and weaknesses of each type of approach.
- Use the collated dataset to understand:
    - If different types or classes of antimicrobials, or individual antimicrobials, are under/overrepresented in the literature.
    - The breadth of different approaches used to generate these data.
    - The approaches in terms of their utility and potential applicability to assessing the potential risk of selection for resistance in the environment as well as their scientific rigour.
    - If there are any general patterns in relation to available MSC/PNECR data according to type of approach.
    - If there are sufficient data for any antimicrobials to have sufficient confidence to propose a robust PNECR at this time.
- Given all the above, highlight knowledge gaps and provide recommendations for future research in this area.

3. Methods

3.1. Generating the database

3.1.1. Literature search

To obtain an understanding of the current MSC/PNECR data available and the approaches used to determine them, a semi-systematic literature search was conducted using the PubMed database. Search terms were generated, then tested to verify they captured key known publications. Following this, the search terms were refined. They 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 Appendix 1. The titles and abstracts for papers identified using these search terms were downloaded and screened for relevance using the criteria in Table 1 to determine inclusion/exclusion.

Table 1. Inclusion and exclusion criteria applied to publications identified in the literature search.

	Inclusion	Exclusion
Compounds	Papers which appeared to generate novel MSC or PNECR data for any antimicrobial, including: antibiotics, antifungals, biocides, metals. 'Antimicrobials' was included as an umbrella term intended to cover antivirals, antiparasitics, antibiotics and antibacterials.	N/A
Organisms	Papers which appeared to generate novel MSC or PNECR data using any method, in any organism. No specific microorganisms were excluded.	Papers investigating organisms other than microorganisms, as they were out of scope for this project
Study type	Papers generating novel MSC/PNECR data.	Papers which only cited previously published MSC/PNECR data, i.e., no new data were generated. These original data sources were checked to ensure all were identified by the search terms, and included.
Resistance type	Papers investigating selection for resistance including chromosomal or mobile (e.g., plasmid-borne) resistance	Papers which only determined concentrations at which *de novo* resistance emerged (see 'Section 5.1.4).

3.1.2. Database curation

After screening of the title and abstract, full texts were downloaded and screened based on the same criteria as above (Table 1). In addition, data were excluded at full text if:

- Data were reported inaccurately (e.g., inconsistencies in reported values in different sections of the paper) or unclearly.
- Papers/data points only included consideration of combinations or mixtures of antimicrobials or antimicrobial and adjuvants.
- LOECs were reported in experimental studies, without NOECs.

Data on published MSCs/PNECRs and the methods used to derive them were input into an Excel spreadsheet (Appendix 3). Data extracted from the papers located included:

- Antimicrobial type (e.g., metal, antibiotic, antifungal etc),
- Antimicrobial class (e.g., tetracycline),
- The individual antimicrobial (e.g., oxytetracycline),
- The MSC, LOEC, NOEC endpoint values reported and their respective units, as well as any reported PNECs, their units and associated assessment factors. For simplicity and to facilitate standardisation of data across approaches, MIC-based effect concentrations (i.e., before AF application) were listed as LOECs in the database compiled (e.g., the values derived by Bengtsson Palme and Larsson, 2016).
- Whether selection or persistence was measured or whether this cannot be known from the data reported,
- The genotype i.e., gene or mutation the endpoint values refer to (where known),
- The genetic context (i.e., 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 the endpoints (e.g., qPCR),
- The bioinformatic pipelines and version (if any were used),
- The paper reference and any additional relevant supporting information was noted in '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.

3.1.3. Generating standardised PNECRs

Some publications reported MSCs (derived by fitting a line of best fit through selection coefficients plotted against test concentrations), whereas others reported LOECs, NOECs, and/or PNECRs. In addition, different units were used. Therefore, the data were used to generate standardised PNECRs as follows by one team member:

- MSC and experimental NOEC data had an AF of 10 applied to generate PNECRs. Modelled data (listed in the LOEC column of the spreadsheet) 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.
- 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.

- Where PNECR data were reported in a publication, the assessment factor was noted. If a different AF to 10 had been used, the PNECR was multiplied by the assessment factor used in the publication before being divided by the assessment factor of 10 used in this study. This standardisation was done to make data comparable, rather than large variation potentially being introduced by use of different assessment factors, for which there are no standardised approaches/recommendations at this time.
- All standardised PNECRs were converted to µg/L.

The standardised column was double checked for accuracy by a second team member.

Standardised PNECRs were compared across types of approaches and systems, including experimental PNECRs vs MIC-based PNECRs, single species vs community experiments, and PNECRs generated using phenotypic or genotypic data.

4. Results

Figure 4 summarises the number of publications/data sources identified from the initial searches, how many remained after screening, and the number of standardised PNECRs that were generated based on the data located (n = 331).

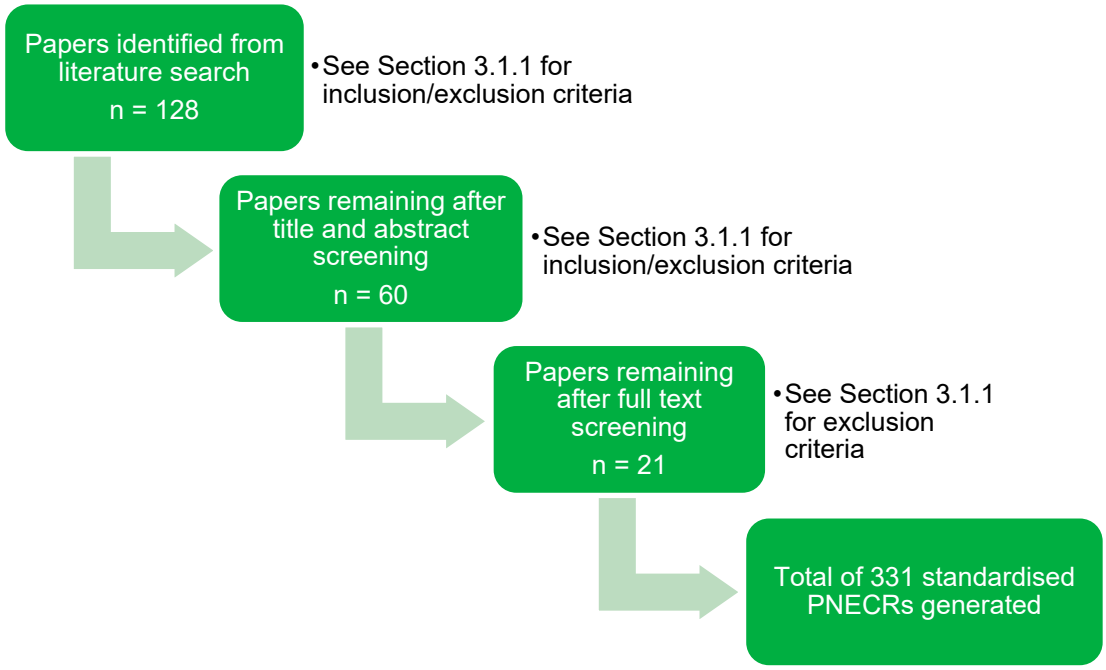

Figure 4. Flow diagram showing the number of publications/data sources identified by semi-systematic searches, those remaining after title/abstract screening and full text screening, and the total number of standardised PNECR data entries generated, based on the data located.

Of the 331 standardised PNECRs collected, 319 were for antibiotics. Given the lack of data for other antimicrobials (see Section 4.1), only antibiotics were studied further. The 319 standardised antibiotic PNECRs were then grouped under different classifications. These were:

- 'Experimental' or 'MIC-based' PNECRs. 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). MIC-based PNECRs were classed as any approach which used a dataset of MICs to estimate PNECRs. Note, some PNECRs were not covered by either of these definitions, and so were excluded from the comparative analyses below (Figures 6 - 8). These PNECRs are still recorded in the database, with a note that they were excluded; they are discussed further in Section 4.2.2.
- '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 *E. 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 derived classified as 'experimental' and 151 PNECRs were classified as 'MIC-based'; 25 PNECRs were not classified as either (see 4.3.2).
- 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 & Larsson, 2016).

4.1 Antimicrobials for which information was identified

Antibiotics were the most studied type of antimicrobial, with only 12 of the 331 standardised PNECRs generated belonging to a different antimicrobial class (6 for metals, 5 for antifungals, and 1 for ionophores, see Appendix 4, Table 1). Similarly, within the antibiotics, several had significantly more standardised PNECRs (Appendix 4, Table 2), such as azithromycin (n = 32), ciprofloxacin (n = 24), clarithromycin (n = 28), erythromycin (n = 25), tetracycline (n = 13) and trimethoprim (n = 29). These five antibiotics represent >45% of all available standardised PNECR for antibiotics in this project. Most antibiotics only had one standardised PNECR value. These usually arose from a single paper i.e., Bengtsson-Palme and Larsson (2016), (see Appendix 4, Table 2). Antibiotics with a minimum of two standardised PNECRs are shown in Figure 5.

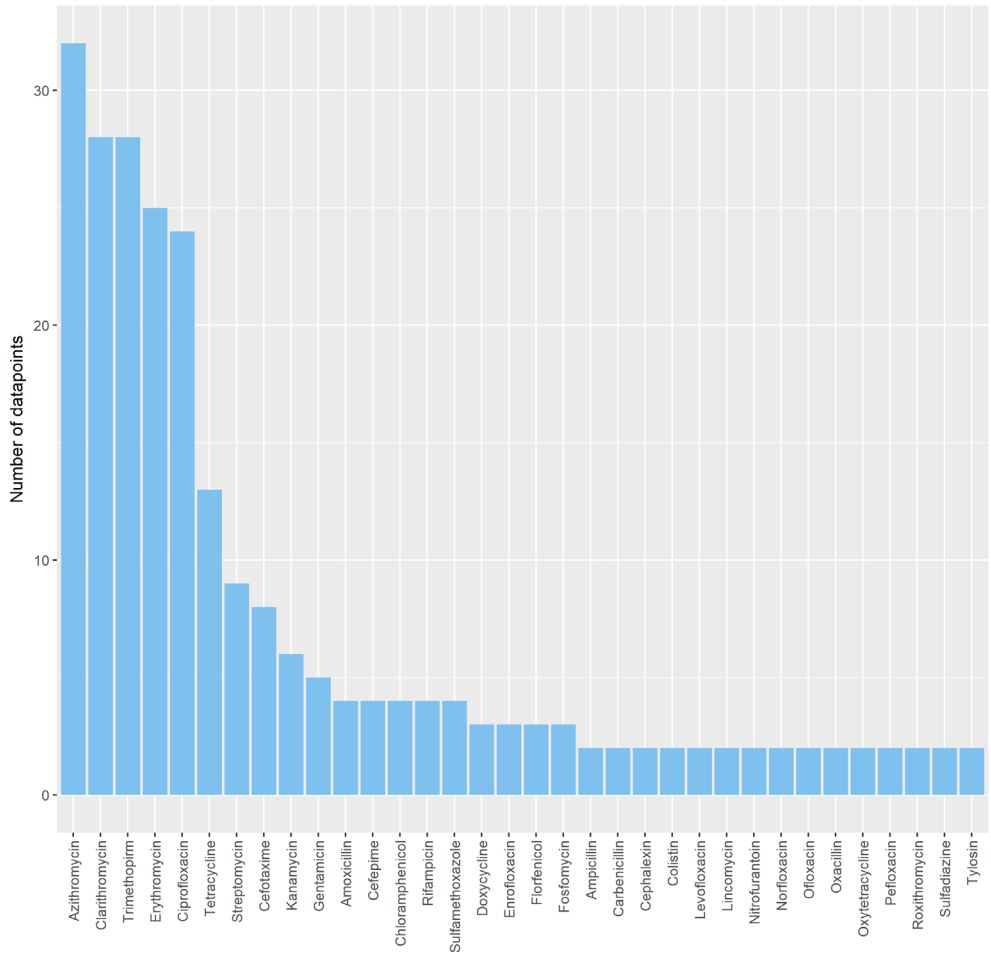

Figure 5. The total number of standardised PNECRs per antibiotic (i.e., includes all data). Only antibiotics with more than one standardised PNECR are shown.

4.2 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 (Figure 5). 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. Conversely ciprofloxacin, trimethoprim, and tetracycline had higher numbers of PNECRs, but these were also from several different studies (Table 2).

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 of 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

4.3 Approaches used to determine MSCs/PNECRs

As well as understanding the availability of MSC and PNECR data across the different antimicrobials and any bias towards certain antimicrobials in the literature, we also considered the different approaches used to generate the data.

4.3.1 Overview of approaches used

A variety of approaches were used to generate the data, and these are discussed in this section, according to the different 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 the 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; Murray et al., 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; Gullberg et al., 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 *Escherichia coli* (Arya et al., 2021; Gullberg et al., 2014; Gullberg et al., 2011; Hjort et al., 2022; 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 testosterone* (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 estimated from growth rate data (e.g., (Klümper et al., 2019)). In complex community studies, a variety of different resistance genes and mutations were quantified, using qPCR and/or metagenomics (e.g., (Lundstrom et al., 2016; Stanton et al., 2020)), although some also used phenotypic 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 used this approach. As mentioned above, the majority of MIC-based PNECRs were from the study by Bengtsson-Palme and Larsson (2016). This study derived 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 wildtype/resistance cut off), and applying an assessment-like factor of 10 to account for the difference between MIC and MSC.

4.3.2 Experimental studies – comparison of standardised PNECRs

The following section compares standardised PNECRs for antibiotics that were 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 (Appendix 2) which did not fit this classification, nor that of MIC-based PNECRs (see Section 4). The PNECRs excluded from these analyses either modelled MSCs from growth rate data for individual species (e.g., Frost et al., 2018; Klumper 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 the most important experimental parameter 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.

In experimental studies, a key question is whether assays using single species 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 test species increased in the presence of the community, compared to when the test species was used in a single species competition experiment (Klümper et al., 2019). Therefore, there is a difficult balance between potentially generating more environmentally representative data and generating fewer protective endpoints using complex communities (as the inherent complexity inevitably increases variance and reduces sensitivity and reproducibility, as well as greater costs and time commitments). Understanding whether community experiments result in higher PNECRs is therefore important for balancing these two different issues.

We compared all single species and community standardised PNECRs across all antibiotics that had at least one standardised PNECR for each inoculum type (trimethoprim, erythromycin, ciprofloxacin and tetracycline, Figure 6). 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 (Appendix 2), 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 persistence of resistance. Therefore, the reason the tetracycline community PNECRs were significantly lower than the tetracycline single species PNECRs may simply reflect that the 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.

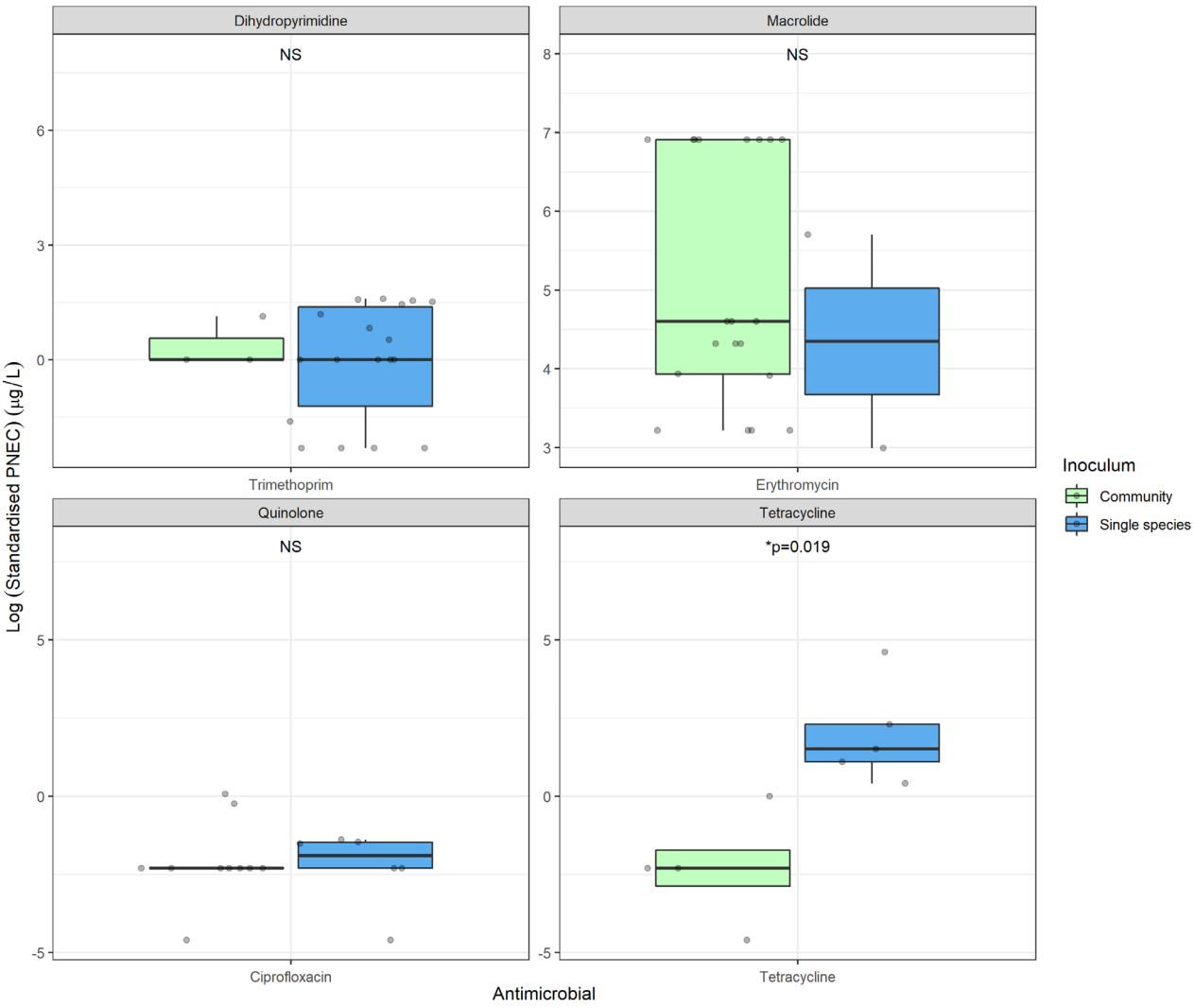

Figure 6. A comparison of experimentally derived PNECRs (standardised) using a single species inoculum or a complex community inoculum. Note, only antimicrobials which have single species and complex community datapoints available are included, and those PNECRs derived using both MSC and NOEC based approaches are shown. Number of PNECRs per antibiotic (community, single species): trimethoprim n=3, n=18; erythromycin n=19, n=2; ciprofloxacin n=13, n=4; tetracycline n=4, n=5. Wilcoxon Rank Sum test used to derive p values. NS = Not significant.

Within the experimental data, phenotypic (i.e., culture based) or genotypic (e.g., qPCR, metagenomics) methods were used to measure resistance endpoints. Generally, it is accepted that phenotypic methods are preferable in that they confirm the resistance phenotype and are cost-effective. However, they can lack sensitivity and specificity compared to molecular, genotypic methods such as qPCR. When examining the standardised PNECRs of antibiotic classes and individual antibiotics (Figure 7), most of the antibiotics only had a single standardised PNECR for either approach, which precluded further exploration. Across all antibiotics with at least one genotypic and one phenotypic PNECR, there was a significant difference between the two datasets ($p = 0.01734$, Wilcoxon Rank Sum test). 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 test). 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 genotypic PNECR).

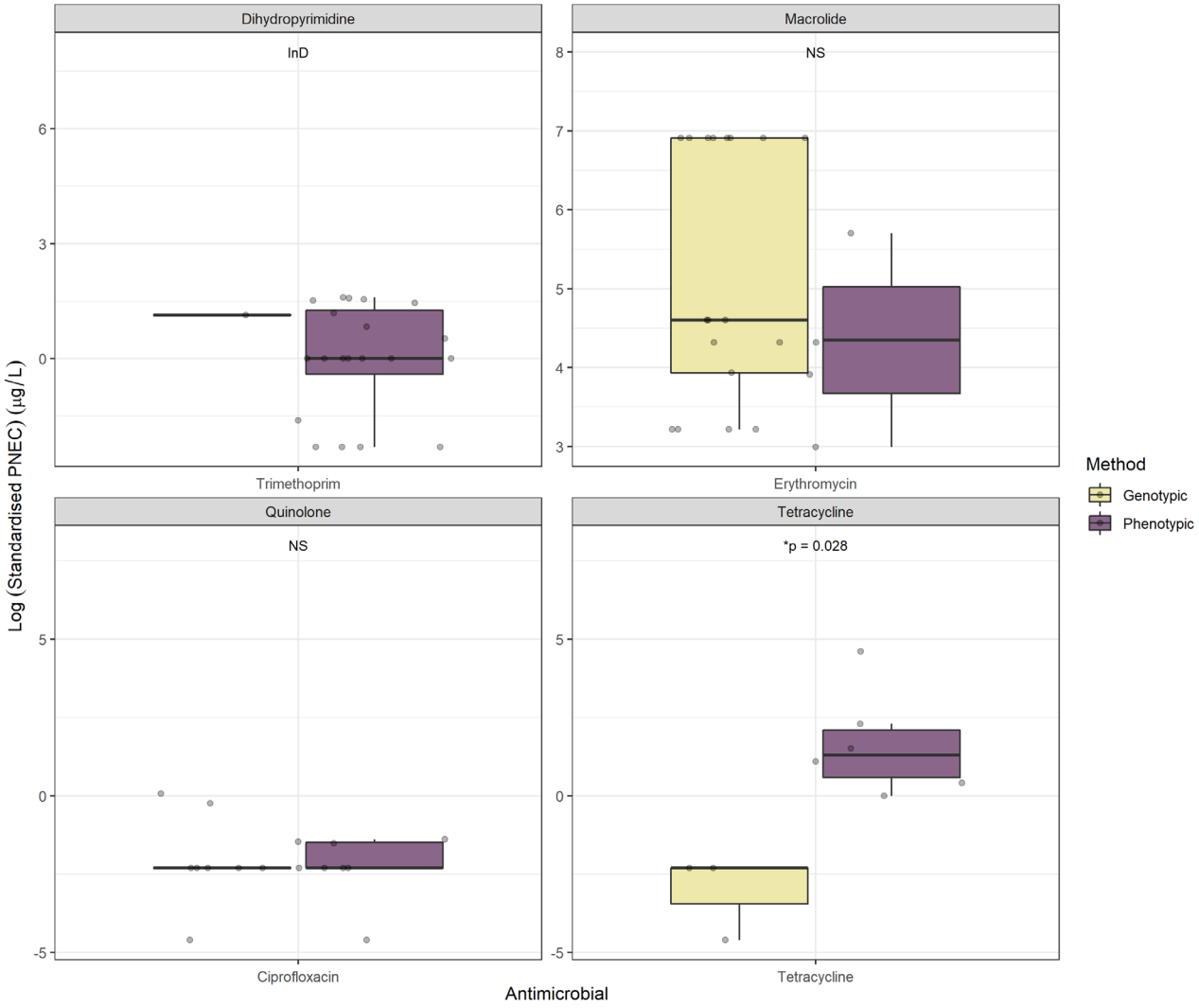

Figure 7. A comparison of standardised experimental PNECRs by the endpoint type (i.e., genotypic, such as qPCR or sequencing; or phenotypic, such as colony forming units/ml), split by antimicrobial compound and class. Note, this shows data from experiments using both types of inoculum (i.e., single species 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, as in, only one standardised PNECR available for one or both methods.

In summary, there are many uncertainties surrounding some of the conclusions represented here, primarily due to lack of data and scientific understanding/consensus. However, these analyses have highlighted these research gaps which can be addressed in future research (see Section 7).

4.3.3 Experimental vs MIC-based PNECRs

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 assessment. Previous comparisons have suggested that PNECRs estimated from MIC data for single species of bacteria are generally more conservative than experimentally derived PNECRs in complex communities of bacteria (Murray et al., 2020). However, there have been several publications not included in this previous analysis (Murray et al, 2020) that have generated MIC-based PNECR data, and many more that have generated experimental data that have not yet been subject to comparison. Indeed, though one of the major benefits of MIC-based approaches is that they can generate significant amounts of data rapidly with a single approach, we have found that the amounts of MIC-based and experimental PNECR data available are almost equal (143 vs 151 standardised PNECRs, respectively). However, experimental studies have tended to focus on a smaller subset of antibiotics and generating several PNECRs for the same antibiotic, whereas MIC-based approaches have generated single PNECR values for a wider range of antimicrobials.

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, Figure 8). 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 gives important insights into risk of AMR evolution in these organisms in environmental settings. However, again, it is not possible to draw any firm conclusions at this time due to insufficient data.

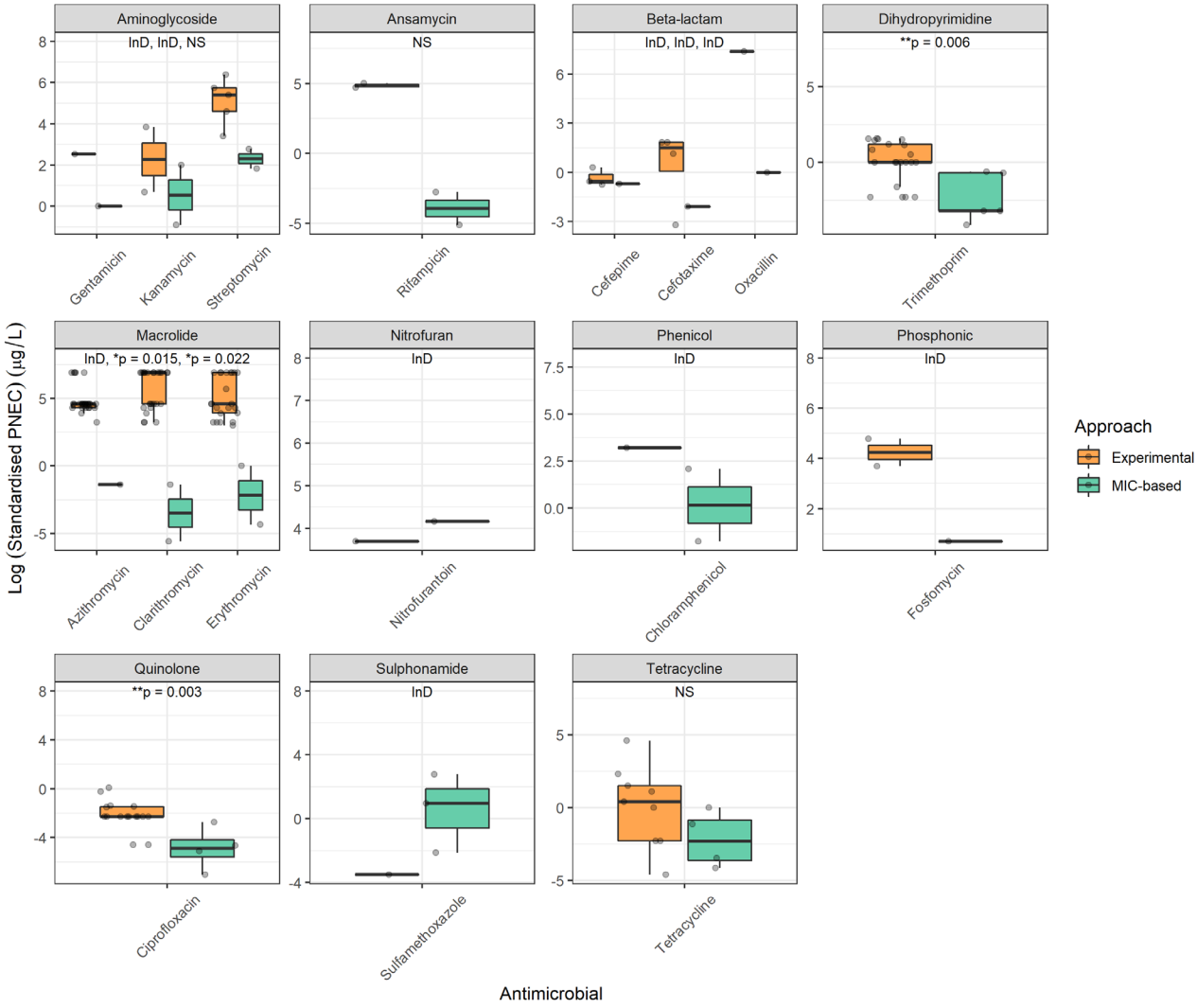

Figure 8. A comparison of experimentally derived standardised PNECRs (i.e., including single species or complex community inoculum, MSC or NOEC based standardised PNECRs, and all genotypic and phenotypic endpoints) and MIC-based PNECRs. Note, only antibiotics and their respective classes with both experimental 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, as in, only one standardised PNECR available for one or both methods.

Further issues to consider when interpreting MIC-based and experimental PNECRs are discussed in the following section. The purpose of this section was to interrogate the data in different ways, to see if any conclusions can be drawn based on the available data. We

suggest at this time, there are still insufficient data available to draw firm conclusions on the following:

- If experimental or MIC-based PNECRs are generally more conservative.
- If genotypic or phenotypic PNECRs/MSCs are generally more conservative
- If community or single species PNECRs/MSCs are generally more conservative.

This lack of data pertains to number of antibiotics tested, as well as the number of times they have been studied and then analysed, in all the various permutations (see next section).

4.4 Considerations when interpreting PNECR data

As noted in Section 1.1, the MIC-based PNECRs generated in the study by Bengtsson-Palme and Larsson (2016) have gained traction as proposed thresholds for a range of antimicrobials. However, during this project, several other studies (Arya et al., 2021; Koutsoumanis et al., 2021; Menz et al., 2019; Rico et al., 2017; Zhang et al., 2022) were identified that generated MSCs/PNECRs using similar approaches. To evaluate and aid the interpretation of the available data, advantages and disadvantages of each type of approach have been listed below and some of these have been discussed previously in (Murray et al., 2021).

4.5 MIC-based approaches – advantages and disadvantages

The advantages of MIC-based approaches are clear in terms of practicality, as they make use of pre-existing MIC data and thus are comparatively much more cost-effective and rapid. In addition, values for a wide range of compounds can potentially be derived with a single approach, which is particularly attractive, as it makes comparisons across antimicrobials simpler when a single method has been applied. This is further enhanced by using data that have been collected according to standardised guidelines, for example MIC data generated through adherence to EUCAST guidelines. In the absence of experimental data, the ability to rapidly generate PNECRs for a variety of compounds, can be used to inform concentration ranges for experiments that aim to determine PNECRs. Studies which also adequately report their methods also facilitate testing the reproducibility of the method, or adaptation of the method as more empirical data emerge. Finally, as the data in this report currently suggest, generally, MIC-based PNECRs may be more conservative and therefore, will offer the greatest protection against selection for AMR in the environment.

However, there are also several disadvantages to MIC-based approaches. Most of these relate to the use of MIC data to derive MSCs/PNECRs, as this does not directly measure competition between resistant and susceptible strains. Some studies make sole use of MIC data from clinical databases, such as EUCAST. This bias towards clinical strains may

generate more or less conservative PNECRs, depending on the MIC distributions. For example, it may be that clinically relevant strains may not survive in the environment, which could skew PNECRs to be more or less conservative depending on whether these strains have lower or higher MICs. To overcome this, some studies have only included data for species that have evidence they can survive in the environment (Tello et al., 2012). Secondly, there is a concern that clinical resistance is on the increase, and therefore over time, MICs would increase, thereby generating less protective PNECRs over time (which is counterintuitive, as more conservative PNECRs should be adopted as the problem exacerbates). This hypothesis could be tested if PNECRs were estimated using archived databases of MICs from previous points in time.

Though it is an advantage to utilise MIC data collected according to published guidelines such as EUCAST, this is also a disadvantage as these recommend high nutrient and high temperature conditions (this is also a common critique against experimental methods as they may not generate environmentally representative data). Furthermore, MIC-based approaches use single species and single antimicrobials, which limits understanding the effect of community on selection (that has been shown to be significant e.g., (Klümper et al., 2019)) and in terms of understanding the effects of complex mixtures of antimicrobials that exist in the environment. Differing AFs could be used in these cases, but there is no discussion/consensus on what these would be. MIC-based approaches also tend to apply a single value to MIC data to derive PNECRs, for example, Bengtsson-Palme & Larsson, 2016 apply a blanket assessment factor-like value of 10 to all antimicrobials. Though AFs may be based on experimental data in some cases (e.g., (Koutsoumanis et al., 2021)), it is clear from experimental evidence that 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; Gullberg et al., 2011). Though application of a single value to represent the MIC/PNECR ratio is likely to be inaccurate, this could be modified in future iterations as more experimental data emerge. Other potential issues to consider when interpreting MIC-based PNECRs is the relevance of MSCs and persistence inducing concentrations, and there is no way to quantify which of these outcomes MIC-based PNECRs represent.

Whilst discussing these disadvantages, we also considered which (if any) were intractable, and which could be improved in future studies. The bias of using only clinical species data could be addressed in future through generation and inclusion of more MIC data for environmentally relevant species. Similarly, through the generation of more experimental data on the relationship between MIC and MSC, the value used to represent this in models could be fine-tuned in future, e.g., according to antimicrobial class or even individual compound. The rest of the issues discussed above we considered to not be easily improved in future studies.

4.6 Experimental approaches – advantages and disadvantages

For experimental approaches, the main disadvantages relate to their practicality. Experimental PNECRs are more expensive and slower to generate than MIC-based PNECRs. Although there were approximately equal numbers of experimental and MIC-based PNECRs available, for experimental PNECRs, these are skewed towards a handful of antibiotics, limiting the breadth of PNECR data available. These seem to have been biased towards antibiotics which have been highlighted as 'of potential concern' for example those included on EU Water Framework Directive Watch Lists (Carvalho et al., 2015; Gomez Cortes et al., 2020; Loos et al., 2018).

Several different approaches employed to determine experimental PNECRs, but no effort has been made to standardise these yet (as is also the case with MIC-based approaches). This, alongside the ability to incorporate complexity, for example by using communities (which is 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 extent of these two concerns.

Other disadvantages associated with experimental approaches were identified and these are discussed below, however, the majority of these could be improved in future studies, with none being intractable. However, this would require significant funds to run not only more studies, but more expensive studies that may measure more effects on more genes or species in more environmentally relevant conditions. For example, most studies to date have used high temperature and high nutrient experimental conditions, which like MIC-based PNECRs, may not generate environmentally representative PNECRs. However, this can be modified through experimental design, and this could also be a criticism of many standardised ecotoxicological assays which are already in use.

Another issue is that some 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, many studies determined PNECRs of antibiotics using environmentally relevant communities of bacteria (e.g., (Kraupner et al., 2020; Murray et al., 2020; Stanton et al., 2020)). 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 active against Gram-positive bacteria) and the depth of data generated available (e.g., in some cases, metagenomics has been used to identify PNECRs for every antibiotic resistance gene that is selected for (Stanton et al., 2020)). This raises a concern of data saturation – there are so many different genes and mutations which could be positively selected, complicating decisions regarding endpoints used to generate PNECRs. However, this resolution and amount of data can be used to generate PNECR ranges, or to define PNECRs based on resistance genes of 'greater concern' (as discussed above); or, simply, to lend further confidence in any definitive PNECR that was chosen. An important caveat with regards to metagenomics/molecular

methods like qPCR is that detection of a given genotype (e.g., presence of a resistance gene) does not necessarily translate into detection of a resistance phenotype (e.g., the gene may be present, but not expressed). Using both culture based and culture independent approaches moving forward would provide confirmation of phenotype, whilst also providing greater depth and sensitivity of data.

In general, for experimental studies, the main advantages we defined were as follows. Experimental studies that aim to determine MSCs/PNECRs are specifically designed to that end, rather than repurposing data collected for another use. This means that they are bespoke, but also flexible, with the experimental design evolving in line with available technologies/techniques and crucially, in line with current scientific understanding. In addition, another significant advantage of experimental studies is that their ability to further understanding can, in turn, inform and improve MIC-based approaches. Experimental approaches have also given insights into the effects of community context on the MSC, social interactions between bacteria with enzymes excreted by resistant strains protecting susceptible cells, and critically they also produce data on the magnitude of selective effects on multiple resistance endpoints in addition to the threshold at which selection occurs.

Many of the intractable issues associated with MIC-based approaches are not found in experimental assays. For example, introducing complexity (such as community effects and mixture effects), tailoring experimental conditions to be more environmentally realistic, confirming whether selection or persistence is observed and providing high resolution data that can be used to interpret relative risk of AMR, can all be incorporated into the experimental design. Finally, unlike MIC-based PNECRs which may become less conservative over time, experimental PNECRs may become more conservative and accurate as science progresses and as technologies and expertise become more accurate and expansive.

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 these PNECRs are invaluable when faced with a lack of experimental data. In addition, MIC-based PNECRs rely on having (e.g., MIC) data available; these are lacking for antimicrobials other than antibiotics and tend to be biased towards MICs in clinical strains of bacteria. Experimental approaches have the potential to be fully optimised, but this is yet to be fully realised in practice and the breadth of experimental PNECR data available remains comparatively limited. Positive feedback should be an objective, where MIC-based PNECRs inform experimental data when they are lacking, and experimental data are used to fine-tune MIC-based PNECRs in the future.

Table 3. Pros and cons of PNECRs generated using an experimental or MIC-based approach. Some of these are raised in (Murray et al., 2021). *(Klümper et al., 2019).

Approach	Pros	Cons
Experimental evolution studies, that measure changes in resistance levels	• Flexibility with experimental design, to design experiments fit for purpose (e.g., directly measuring increases in resistance). • Several studies use complex communities, therefore more environmentally representative and capture important ecological interactions which influence the MSC*. • Can use more environmentally representative test conditions. • Can be used to test mixture effects in future. • Experimental data are needed to generate better MIC-based approaches. • Differences are determined using statistical tests, more in line with current environmental risk assessment approaches. • Can measure persistence vs selection. • As scientific methods/technologies improve, PNECRs should only be come more protective as techniques become more sensitive.	• Significantly more costly and time-consuming. • Lack of standardisation across assays. • Higher variation and potentially lower reproducibility, particularly when using communities due to the complexity and potential founder population issues. • Often use high temperature and high nutrient conditions, which are not environmentally representative. • Often only genotypic endpoints are measured, which may not translate into a change in phenotype. • Single species experiments lack community and ecological effects known to influence the MSC*. • Few compounds have been studied thus far. • Lack of consensus on the best endpoint to measure (e.g., which gene/host?) and effect of most interest (e.g., persistence or selection?).
MIC-based	• Use previously generated data, so quicker and more cost-effective. • Use data generated using standardised approaches, so MIC data are directly comparable. • Related to above, can incorporate large amounts of data. • Reproducible and can be easily repeated when more data become available.	• Usually biased towards clinical strains which may not survive in the environment. • Tend to use a single factor to represent MIC:MSC ratio, which can vary even for the same compound for different resistance mechanisms. • Use of single strains grown in isolation precludes any community/ecological interactions, which are known to impact MSCs *.

Approach	Pros	Cons
	• Can be improved as more experimental data are generated (e.g., to inform MIC/MSC relationship). • Only option when there are no experimental data available. • Results can be used to inform experiments, e.g., indicative concentration ranges. • Lots of PNECR data generated using a single approach.	• MIC data collected under high nutrient and temperature conditions, which are not environmentally realistic. • Inability to test selective effects of mixtures in current approaches. • Unable to distinguish/measure persistence vs selection. • Using data on susceptibility to infer selection potential – MIC data are not collected for this purpose. • Concerns that in future, if AMR increases, MICs will increase, therefore future iterations may generate higher PNECRs over time.

5. Discussion

5.1 Knowledge gaps

This report has identified several knowledge gaps which should be explored with further research. These are discussed below.

5.1.1. Antimicrobials other than antibiotics

The semi-systematic search identified a total of 331 MSC/PNECR data points, 319 of which were for antibiotics. The 11 non-antibiotic data points were for metals, antifungals and one ionophore. Metals can co-select for AMR (Baker-Austin et al., 2006), and antifungals may select for antifungal resistance at environmentally relevant concentrations (Jeanvoine et al., 2020; Snelders et al., 2009; Verweij et al., 2009). Further studies should aim to generate data on selection for resistance for other antimicrobials.

In two Environment Agency research projects, concentrations of antifungals which may influence selection for resistance have been considered. One project (Environment Agency, 2024a) generated PNECRs for a number of clinical antifungals, based on the method developed by Bengtsson-Palme and Larsson (2016), using MIC data collected from literature searches. Although agricultural fungicides were also considered, PNECRs could not be determined as the MIC data were lacking. Another project explored the development of a simple single (yeast) species competition assay to MSCs for a clinical and an agricultural antifungal (Environment Agency, 2024b).

Generating effects data, and subsequently PNECRs, for antifungals is important and should not be overlooked when considering the role of the environment in the selection of AMR. A large proportion of antifungal substances used are applied directly to soils as plant protection products (Garthwaite et al., 2018), and, as a result, antifungal resistance has been found in crop pathogens, which poses risks to both 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 originated 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 a significant body of evidence indicating that biocides can co-select for antimicrobial resistance, yet no PNECRs for these exist. Evidence is emerging that other classes of compounds, for example, non-antibiotic pharmaceuticals (Maier et al., 2018; Y. Wang et al., 2022; Wang et al., 2023) and plant protection products may also play a role in AMR selection and dissemination (Kurenbach et al., 2015; Liao et al., 2021). Therefore, it is likely the range of compounds that will need to be considered will continue to expand as research progresses.

5.1.2. Complex mixtures of antimicrobials

Further to the lack of PNECR data for individual antimicrobials and potentially co-selective compounds, there is also very limited understanding of how these compounds may interact in complex mixtures. This is an issue relating to the assessment of the impact of chemicals on the environment more generally, and not just in relation to the assessment of the impact of selection for resistance.

In this report, we excluded the few MSC/PNECR data available for combinations of antibiotics with metals or antibiotic adjuvants, as the data were too few to draw any conclusions, and this was not in scope of this analysis. Though some antibiotic-adjuvant combination PNECRs have been estimated previously (Bengtsson-Palme & Larsson, 2016), it is a limitation of current approaches that use MIC data, as the latter measure effects of individual antimicrobials in isolation. Furthermore, extrapolating PNECRs that reflect complex mixture interactions will be challenging given the scarcity of MIC data for antimicrobial compounds such as biocides and metals. 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 (Rodea-Palomares et al., 2015). However, one study showed that 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.

One approach that could be used in the interim is 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 PNEC, 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. Though this lack of a defined upper boundary is a barrier to implementing MAFs through an aversion to being too conservative, 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. 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 for REACH and noted a potential MAF of 5 (EA, 2022). All these recommendations have been developed in relation to consideration of impact of chemicals in general and therefore their relevance for use in relation to assessment of selection for resistance would need to be considered.

In summary, understanding of mixture effects of antimicrobials and co-selective compounds remains limited by lack of experimental studies on mixture effects; lack of PNECR and MIC data for individual antimicrobials generally; and incomplete understanding of compounds that could co-select for AMR, which could require PNECR data. Consideration of approaches being developed to consider mixtures, e.g., MAFs would be needed applicability to different environments.

As discussed, both MIC-based and experimental PNECRs tend to be generated using artificial, laboratory conditions (either by using MIC data collected under these conditions, or in selection/evolution experiments using these conditions). 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 model sediment and soil PNECRs from these by considering how these chemicals behave in soil environments. This was performed recently for antifungal PNECRs that were generated using a modified version of the Bengtsson-Palme and Larsson (2016) approach (Environment Agency, 2024a), but was beyond the scope of this project.

There is also a debate over whether experimental PNECRs are relevant to the environment, given they were generated under laboratory conditions. 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. These issues relate to all ecotoxicity tests and are not specific to AMR.

Furthermore, there is some evidence that different experimental conditions may have limited impacts on the PNECR. For example, one study (Murray et al., 2020) used artificial sewage growth media (as recommended in the OECD approved activated sludge respiration inhibition test (OECD, 2009)), and reduced the temperature to ambient (21°C ± 2°C), which is more representative of environmental conditions. 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).. A further study by 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).

5.1.3. 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.

6. Recommendations

6.1 Standardisation within studies – challenges and opportunities

Many different approaches have been used to generate PNECRs, creating difficulties when trying to compare PNECR data for individual antibiotics across studies. Experimental studies rarely use the same system to study large numbers of antibiotics, or directly compare PNECRs for a single compound generated with different approaches – though there are some exceptions (e.g., (Kraupner et al., 2020; Murray et al., 2020; Stanton et al., 2020)).

There are significant obstacles that would need to be overcome to generate a standardised assay. These extend beyond the requirement of ring trials and confirming intra- and inter-laboratory reproducibility. Decisions need to be made on the following aspects of PNECR determination.

1. **The effect endpoint, and how it should be measured, is still undecided**. As mentioned above, metagenomic studies can generate multiple PNECRs for all resistance genes detected that increase in relative abundance. High-throughput qPCR assays can also generate large amounts of data, without a decision being made 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. One approach is to use the class 1 integrase gene as a measure of selection for genetic platforms (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). Even for qPCR assays, there would ideally be some standardisation of reagents, reactions, programmes, and primers across studies, which is yet to be realised. 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. Studies only using phenotypic data overlook the reservoir of AMR that exists in unculturable bacteria, which may be better represented by culture independent approaches. Some suggestions on prime 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. There is also no consensus on which endpoint (e.g., MSC, LOEC, NOEC, or others) is preferred, and why.

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

increased assessment factors (AFs) to PNECR data. What these AFs may be (both for individual compounds and in terms of MAFs) requires further discussion.

3. **Experimental studies use different species, or mixtures of species (communities), isolated from the clinic, laboratory or different environments**. Within these, there are close to infinite possibilities of the resistance mechanisms/genes which could be studied, with genetic contexts and hosts that can also widely vary. All of these can impact the value of the effect endpoint and it is not feasible to explore all options to find the most conservative approach in each scenario. 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 likely to result in less conservative PNECRs than MIC-based approaches. However, if the objective is to understand selection for AMR in wastewater or environmental microbiomes, MIC-based approaches have their own limitations. A refined assessment factor that accounts for the uncertainty arising from using communities could also be used, but how this assessment factor would be determined is beyond the scope of this review.

4. **Consideration of the outcome that should be measured is required**. We have already discussed this in terms of measuring selection or persistence in the Introduction (Section 1.3). In addition, there is a question over whether regulatory endpoints should be based solely on concentrations where resistant strains are enriched over susceptible strains; or whether they should also consider the lowest concentration at which resistant strains are created (i.e., where selection for *de novo* mutation occurs). We did not include any studies that only determined concentrations at which novel resistance mutations emerge, as this was beyond the scope of the project. Though not reported here, most studies in this space define concentrations which select for novel resistance mutations, without defining the lowest concentration that selected for that novel resistance. 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 and apply these to environmental settings.

Though lack of standardisation is in some ways, a hinderance, it also offers a significant benefit. Given all the factors that vary between studies that could impact PNECRs, it is quite remarkable that PNECRs across studies can be so similar in some cases (e.g., for ciprofloxacin, Figure 6 & Figure 7). Due to the diversity of approaches applied, it can be argued that there should be greater confidence in similar PNECRs generated with different approaches, rather than identical PNECRs generated with a single approach which may not be fully optimised.

6.2 Refining existing approaches to further understanding

During our exploration of the PNECR data, we identified several opportunities for furthering our understanding by using existing approaches with different datasets.

1. **Application of different cut-off values.** Some models have taken the 1% or 5% MIC values (or HC5% value, in some cases) and used these to generate MIC-based 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). We suggest that calculations are rerun using these different values, to determine whether they make any material difference to 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 models should be recommended.

2. **Assessment of relative contribution of Gram-positive and Gram-negative species.** We suggested that the differences between MIC-based and experimental PNECRs may be in part due to the predominant focus on Gram-negative organisms in experimental studies, whereas MIC-based approaches include MIC data for Gram-positive organisms, that may be more susceptible and have lower MICs. We therefore propose that MIC-based approaches could be repeated using only MICs for Gram-negative bacteria and only MICs for Gram-positive bacteria, to see if PNECRs generated substantially differ, and if PNECRs for Gram-positive bacteria are lower. This could inform future experimental studies and debate around relative risk of resistant Gram-positive and Gram-negative bacteria in the environment. A different assessment factor for each group could also be applied, though how this would be defined is beyond the scope of this review.

3. **Exploration of temporal effects of datasets.** We suggested in Section 4.5 that one of the issues with MIC-based approaches is that the PNECRs could become less protective overtime if resistance continues to increase, as this would mean the MICs used to generate the PNECRs would also increase. This hypothesis could be rejected if calculations were repeated using archived datasets e.g., from 10, 20 years ago, and PNECRs were not reduced compared to when more recent data were used. However, this would need to be considered carefully. It would be necessary to analyse a subset of current data, as archived datasets may have less data available (e.g., number of species with MIC data), so it would be important to compare like for like, or to control for differences in dataset size.

4. **Expansion of MIC datasets.** Finally, a more long-term recommendation is to focus efforts on generating 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 MIC dataset that would generate PNECRs

that are more representative of the environment, and for a wider range of co-selective antimicrobials which may have different uses. For example, a recent study that determined PNECRs for antifungals using an approach based on the Bengtsson-Palme and Larsson (2016) methodology was limited to determining PNECRs for a small range of clinical antifungals, as there were limited MICs found in the literature (unable to satisfy the data requirements for the modelling) for all agricultural antifungals and for some of the clinical antifungals of interest (in some cases, no MIC data was found) (Environment Agency, 2024a). These values are not included in our PNECR database, as this was not published when the search was undertaken.6.3 Establishment of definitive PNECRs

6.3 Establishment of definitive PNECRs

As discussed previously, there are inherent difficulties in assigning 'definitive' PNECRs, relating to the complexity of AMR in microbiomes. For antibiotic resistance, which most of the available PNECR data pertain to, this results from the fact that resistance can exist in, and be transferred between, many different species and communities which vary significantly at the genetic (e.g., type of resistance, genetic context of resistance) organism, community, and environmental scale. The diversity of potential selection endpoints that could be measured in experimental studies is vast and vary in terms of AMR risk (e.g., if the genes are mobile and can easily spread between species and/or confer resistance to critically important antibiotics).

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. We suggest 0.01 µg/L for several reasons. 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 important to note that this is an incomplete, and in many cases, a 'shallow' dataset – i.e., many antibiotics only have a single PNECR available. We cannot make specific recommendations for other antimicrobials as there are insufficient data at this time.

7. Summary of recommendations for future work

The main aim of this project was to collate available data relating to the approaches that have been used to determine concentrations at which selection for resistance occurs, the values derived and present current understanding. Considering the data collected and our interpretation of them, we suggest the following recommendations for future (research) work:

- The PNECR data reported should be used in combination with predicted or measured environmental concentration data at high temporal and spatial resolution to generate a clear picture of the extent and severity of AMR selection risk in the environment.
- Research should move beyond the study of selection for antibiotic resistance, to encompass antifungal resistance and AMR more holistically (i.e., through study of selection by metals, biocides, and other co-selective compounds).
- A value of 0.01 µg/L could be applied for all antibiotics until evidence emerges that this is not conservative enough. This is similar to, but slightly lower than, the default PNEC of 0.05µg/L recommended by the AMR Industry Alliance (Vestel et al., 2021).
- Understanding of complex mixture effects is crucial and should be considered (and is not reflected in the blanket value suggested above).
- The database of endpoints reported, eg MSCs/PNECRs will not be exhaustive as a fully systematic approach was not adopted. This could be performed in future to generate a more comprehensive database. Appraisal of data quality could also be performed in future.
- Existing data and approaches can be further explored and repurposed to gain further understanding of differences in and reliability of PNECRs already generated, e.g., rerunning MIC-based calculations as and when more data become available (See Section 4.5).
- The database provided here can be used to prioritise research on compounds where data are currently lacking, in combination with data on those which are mostly widely used, at the highest volumes, and those which are most persistent in the environment.
- Sediment and soil PNECRs could be modelled using the PNECRs described here and used to understand potential risk of AMR in different environments.
- Regulators, policy makers and other relevant stakeholders should engage in discussions around data requirements (i.e., around standardisation requirements, endpoints, appropriate assessment factors and outcomes such as selection/persistence and *de novo* selection) to help direct future study.

8. Conclusions

In this report, we collated MSC and PNECR data for antimicrobials with corresponding metadata regarding the approaches used to derive them. Using this data, we were able to identify compounds for which no MSCs or PNECRs exist, as well as compounds for which PNECR/threshold concentration ranges could be indicated. This database could be expanded with a fully systematic search and added to over time as new data emerge. In addition, it could be used as a resource to direct future research efforts that focus on addressing the most pressing data gaps. We highlighted several of these, and suggested how these might be addressed in the immediate and long term.

In addition, we discussed the relative advantages and disadvantages associated with MIC-based and experimental approaches that generate PNECRs. At this time, both approaches have their merits, particularly as data continue to emerge in this area. We also highlighted several issues unique to risk assessment of AMR that require discussion, such as problems with standardisation, trade-offs between approaches, and the preferred outcomes for environmental regulation.

In conclusion, this report is a comprehensive summary and discussion of PNECR data for (predominately) antibiotics, that can be used to inform decision making around thresholds for compounds that risk selecting for AMR in the environment. The complexities surrounding the generation of PNECRs, and their interpretation have also been outlined and discussed. Currently, there are insufficient data to draw firm conclusions around which approach(es) produce the most accurate or informative estimates.

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10. List of abbreviations

Abbreviation	Explanation
AMR	Antimicrobial resistance
EQS	Environmental quality standard
LOEC (s)	Lowest observed effect concentration
MEC (s)	Measured environmental concentration
MIC (s)	Minimum inhibitory concentration
MSC (s)	Minimal selective concentration
NOEC (s)	No observed effect concentration
PEC (s)	Predicted environmental concentration
PNEC (s)	Predicted no effect concentration
PNECR (s)	Predicted no effect concentration for [antimicrobial] resistance
qPCR	Quantitative real-time polymerase chain reaction

Appendix 1 - Search strategy

Known includes

Publications that were known to be relevant to this report by the team prior to the searches being undertaken were:

1. Bengtsson-Palme J, Larsson DG. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ Int. 2016 Jan;86:140-9. doi: 10.1016/j.envint.2015.10.015. Epub 2015 Nov 17. PMID: 26590482.
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4. Hjort K, Fermér E, Tang PC, Andersson DI. Antibiotic Minimal Selective Concentrations and Fitness Costs during Biofilm and Planktonic Growth. mBio. 2022 Jun 28;13(3):e0144722. doi: 10.1128/mbio.01447-22. Epub 2022 Jun 13. PMID: 35695458; PMCID: PMC9239065.
5. Klümper U, Recker M, Zhang L, Yin X, Zhang T, Buckling A, Gaze WH. Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. ISME J. 2019 Dec;13(12):2927-2937. doi: 10.1038/s41396-019-0483-z. Epub 2019 Aug 5. PMID: 31384011; PMCID: PMC6864104.
6. Kraupner N, Ebmeyer S, Bengtsson-Palme J, Fick J, Kristiansson E, Flach CF, Larsson DGJ. Selective concentration for ciprofloxacin resistance in Escherichia coli grown in complex aquatic bacterial biofilms. Environ Int. 2018 Jul;116:255-268. doi: 10.1016/j.envint.2018.04.029. Epub 2018 Apr 25. PMID: 29704804.
7. Kraupner N, Ebmeyer S, Hutinel M, Fick J, Flach CF, Larsson DGJ. Selective concentrations for trimethoprim resistance in aquatic environments. Environ Int. 2020 Nov;144:106083. doi: 10.1016/j.envint.2020.106083. Epub 2020 Sep 2. PMID: 32890888.
8. Lundström SV, Östman M, Bengtsson-Palme J, Rutgersson C, Thoudal M, Sircar T, Blanck H, Eriksson KM, Tysklind M, Flach CF, Larsson DGJ. Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms. Sci Total Environ. 2016 May 15;553:587-595. doi: 10.1016/j.scitotenv.2016.02.103. Epub 2016 Mar 22. PMID: 26938321.
9. Murray AK, Zhang L, Yin X, Zhang T, Buckling A, Snape J, Gaze WH. Novel Insights into Selection for Antibiotic Resistance in Complex Microbial Communities. mBio. 2018 Jul 24;9(4):e00969-18. doi: 10.1128/mBio.00969-18. PMID: 30042197; PMCID: PMC6058293.

10. Murray AK, Stanton IC, Wright J, Zhang L, Snape J, Gaze WH. The 'SELection End points in Communities of bacTeria' (SELECT) Method: A Novel Experimental Assay to Facilitate Risk Assessment of Selection for Antimicrobial Resistance in the Environment. Environ Health Perspect. 2020 Oct;128(10):107007. doi: 10.1289/EHP6635. Epub 2020 Oct 21. PMID: 33084388; PMCID: PMC7577113.
11. Stanton IC, Murray AK, Zhang L, Snape J, Gaze WH. Evolution of antibiotic resistance at low antibiotic concentrations including selection below the minimal selective concentration. Commun Biol. 2020 Sep 3;3(1):467. doi: 10.1038/s42003-020-01176-w. PMID: 32884065; PMCID: PMC7471295.
12. Vos M, Sibleyras L, Lo LK, Hesse E, Gaze W, Klümper U. Zinc can counteract selection for ciprofloxacin resistance. FEMS Microbiol Lett. 2020 Feb 1;367(3):fnaa038. doi: 10.1093/femsle/fnaa038. PMID: 32105320; PMCID: PMC7082703.

These known includable publications were used to test search terms to ensure search terms designed were identifying these relevant publications.

Search terms

After testing multiple search term iterations, it was decided to use two sets of search terms to ensure all known includable publications were captured. These terms, along with the known includes identified and the number of search hits found in PubMed can be found in Appendix 1 Table 1. The searches were de-duplicated against each other using EndNote X8 to remove multiple copies of the same publication.

Appendix 1 Table 1. The two search strategies used in PubMed. Search terms are split by category and where multiple search categories were used in one search string (search number 1), an “AND” term was used between different categories. The numbers listed in the “Known includes found” column relate to the number of the publication in “Known includes” section above.” “Hits” refers to the number of publications found for both searches in PubMed.

Search number	Search term category	Search terms	Known includes found	Hits
1.	Selection	"MSC" OR "minimal selective concentration*" OR "minimum selective concentration*" OR "minimum selection concentration*" OR "PNEC*" OR "predicted no effect concentration*" OR "selective concentration*" OR "selection concentration" OR "minimal selection concentration"	1, 3, 4, 5, 6, 7, 8, 10, 11, 12	95
	Antimicrobials	"antimicrobial*" OR "antibiotic*" OR "antifungal*" OR "biocide*"		
	Resistance	"AMR" OR "antimicrobial resistan*" OR "antibiotic resistan*" OR "biocide resistan*" OR "antifungal resistan*"		
2.	Selection	low antibiotic* AND selection	2, 4, 5, 9, 11	40
	Antimicrobials	N/A		
	Resistance	N/A		

Appendix 2 – PNECR dataset

See accompanying Excel spreadsheet, “SC220007_PNECR dataset”.

Appendix 3 – Data entries by antimicrobial class and per antimicrobial substance

Appendix 4 Table 1. Number of standarised PNECRs (n) by antimicrobial class.

Class	n
Aminoglycoside	27
Ansamycin	4
Anti-TB	3
Azole	3
Beta-lactam	53
Carbapenem	4
Carboxylic acid	1
Cyclic lipopeptide	1
Dihydropyrimidine	30
Echinocandin	1
Fusidane	1
Glycopeptide	2
Ionophore	1
Ketolide	1
Lincosamide	3
Macrolide	91
Metals	6
Nitrofuran	2
Nitroimidazole	1
Orthosomycin	1
Oxazolidinone	1
Peptide	1
Phenicol	8
Phosphonic	3
Pleuromutilin	2
Polyene	1
Polymyxin	1
Polypeptide	1
Quinolone	46
Streptogramin	2
Sulphonamide	7
Tetracycline	22

Appendix 4 Table 2. Number of data entries (n) per antimicrobial.

Antimicrobial	n
Amikacin	1
Amoxicillin	4
Amphotericin B	1
Ampicillin	2
Anidulafungin	1
Avilamycin	1
Azithromycin	32
Aztreonam	1
Bacitracin	1
Benzylpenicillin	1
Capreomycin	1
Carbenicillin	2
Cefaclor	1
Cefadroxil	1
Cefalexin	1
Cefaloridine	1
Cefalothin	1
Cefazolin	1
Cefdinir	1
Cefepime	4
Cefixime	1
Cefoperazone	1
Cefotaxime	8
Cefoxitin	1
Cefpirome	1
Cefpodoxime	1
Ceftaroline	1
Ceftazidime	1
Ceftibuten	1
Ceftiofur	1
Ceftobiprole	1
Ceftriaxone	1
Cefuroxime	1
Cephalexin	2
Chloramphenicol	4
Chlortetracycline	1
Ciprofloxacin	24
Clarithromycin	28
Clinafloxacin	1
Clindamycin	1
Cloxacillin	1
Colistin	2
Copper	1
Copper (II) sulfate	1

Antimicrobial	n
Daptomycin	1
Difloxacin	1
Doripenem	1
Doxycycline	3
Enrofloxacin	3
Ertapenem	1
Erythromycin	25
Ethambutol	1
Faropenem	1
Fidaxomicin	1
Fleroxacin	1
Florfenicol	3
Fluconazole	1
Flumequine	1
Fosfomycin	3
Fusidic acid	1
Gatifloxacin	1
Gemifloxacin	1
Gentamicin	5
Imipenem	1
Isoniazid	1
Itraconazole	1
Kanamycin	6
Lead	1
Levofloxacin	2
Lincomycin	2
Linezolid	1
Lomefloxacin	1
Loracarbef	1
Mecillinam	1
Mercury	1
Meropenem	1
Methacycline	1
Metronidazole	1
Minocycline	1
Moxifloxacin	1
Mupirocin	1
Nalidixic acid	1
Narasin	1
Neomycin	1
Netilmicin	1
Nitrofurantoin	2
Norfloxacin	2
Ofloxacin	2
Ormetoprim	1

Antimicrobial	n
Oxacillin	2
Oxytetracycline	2
Pefloxacin	2
Penicillin	1
Phenoxymethylpenicillin	1
Piperacillin	1
Quinupristin–dalfopristin	1
Retapamulin	1
Rifampicin	4
Roxithromycin	2
Secnidazole	1
Silver	1
Sparfloxacin	1
Spectinomycin	1
Spiramycin	1
Streptomycin	9
Sulfadiazine	2
Sulfamethoxazole	4
Sulfathiazole	1
Teicoplanin	1
Telithromycin	1
Tetracycline	13
Thiamphenicol	1
Tiamulin	1
Ticarcillin	1
Tigecycline	1
Tilmicosin	1
Tobramycin	1
Trimethoprim	29
Trovafloxacin	1
Tylosin	2
Vancomycin	1
Viomycin	1
Virginiamycin	1
Zinc	1

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