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Predicting antifungal concentrations that select for resistance: An enhanced approach to establish environmental thresholds

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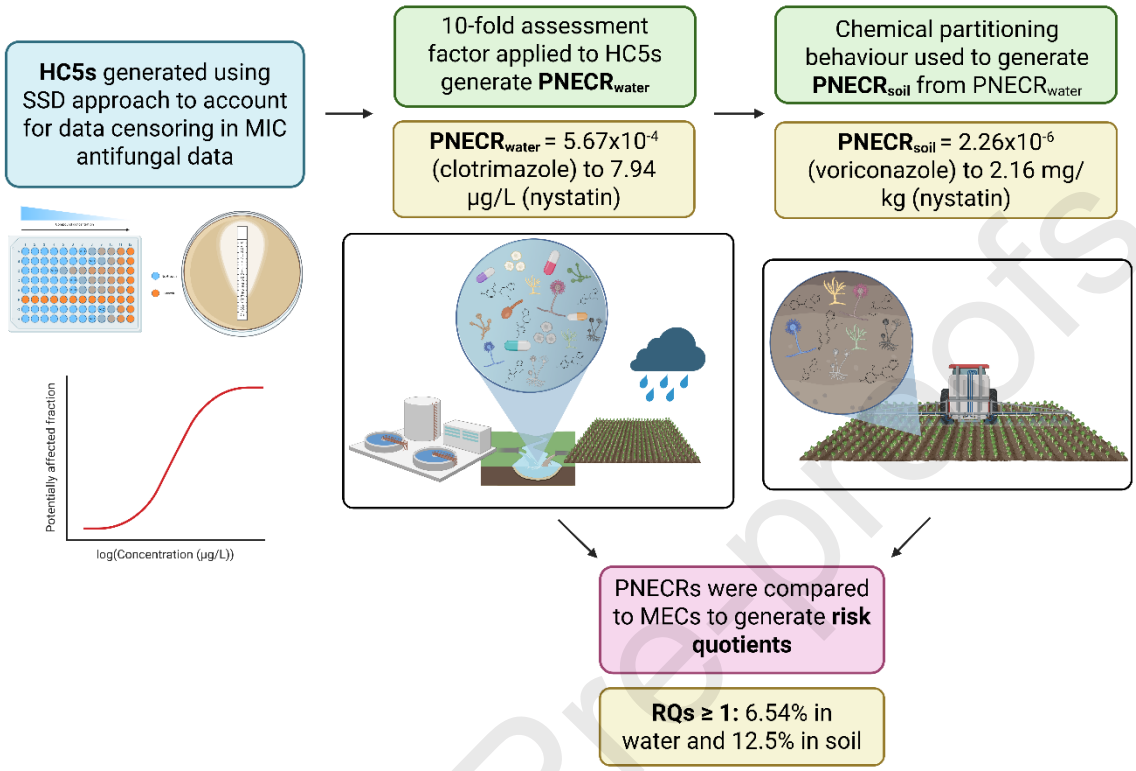
Graphical Abstract



Abstract

Antifungal resistance (AFR) is an emerging threat. Understanding the concentrations at which antifungals select for resistance is critical for guiding policy to minimise risks. This study aimed to determine predicted no effect concentrations for resistance (PNECRs) for antifungals in water and soil. PNECRs for water ($PNECR_{water}$) were derived from species sensitivity distributions fitted using a Maximum Likelihood Estimation approach to estimate the lower 5th percentile Hazard Concentrations (HC5s) from censored species/compound level MIC data and applying a 10-fold assessment factor. PNECRs ranged from $5.67x10^{-4}$ (clotrimazole) to 7.94 µg/L (nystatin). PNECRs derived using standard methodologies that do not account for censoring are always higher, and therefore less conservative for environmental protection, than when considering censoring. PNECRs for soil ($PNECR_{soil}$) were derived by applying soil partitioning coefficients to $PNECR_{water}$ for each antifungal, thereby providing an estimate for the bulk soil concentration needed to achieve the $PNECR_{water}$ in soil pore water. These ranged from $2.26x10^{-6}$ (voriconazole) to 2.16 mg/kg (nystatin). Risk quotients were generated from measured environmental concentrations, and 6.54% for water (n = 200) and 12.5% for soil (n = 1) were over 1, suggesting selection for AFR could be occurring. This type of data generation and analyses will inform discussions about targeted mitigation strategies to reduce the risk of selection for AFR, however, PNECR estimations can be improved with increased data for certain compounds, particularly agricultural fungicides. Preventing an increase in resistance is critical for reducing the risk posed to human health from exposure to environmental AFR.

Key words

Antifungal resistance; AMR; Predicted no effect concentrations; Risk assessment; Antifungals; Environment

Introduction

Antimicrobial resistance (AMR) is a global health threat predicted to cause 10 million deaths per year by 2050 (O'Neill 2014); encompassing resistance in all microorganisms including fungi, bacteria, viruses; and occurs in clinical, agricultural and environmental settings (Singer, Shaw et al. 2016, Fisher, Burnett et al. 2024). Fungi cause a diverse range of human infections, which can result in life-threatening diseases (Fisher, Alastruey-Izquierdo et al. 2022) and the World Health Organisation now recognises fungi as a key risk to human health, publishing a fungal priority pathogens list (World Health Organization 2022). Further, the Centers for Disease Control and Prevention (CDC) recognises the risk of drug-resistant fungi, classifying resistant *Candida auris, Candida spp*. and *Aspergillus fumigatus* as "urgent," "serious threat" and "watch list", respectively (Centers for Disease Control and Prevention 2019). A range of diseases, from asymptomatic to systemic, can be acquired following exposure to environmental fungi (Denham, Wambaugh et al. 2019, Steffen, Smith et al. 2023), therefore, limiting the spread of resistant fungi in the environment is critical to protect human health and ensure that clinical treatments remain effective.

Antifungal compounds can enter the environment from anthropogenic sources (Richter, Wick et al. 2013, Fisher, Hawkins et al. 2018). For example, antifungals are often used in personal care products such as skin creams (National Institute for Health and Care Excellence 2023) and anti-dandruff shampoos (Okokon, Verbeek et al. 2015), which can result in active ingredients entering sewer systems (Peng, Huang et al. 2012, Richter, Wick et al. 2013, Assress, Selvarajan et al. 2019, Assress, Nyoni et al. 2020, Assress, Selvarajan et al. 2021, Wronski, Trawinski et al. 2024). Further, oral antifungals can be excreted as their parent chemical (i.e., active) in both urine and faeces; however, metabolism in the body or biodegradation in the sewer can reduce the load entering wastewater treatment plants (Bellmann and Smuszkiewicz 2017, Carmo, Rocha et al. 2023). As such, topical and oral antifungals can enter municipal wastewater (Lindberg, Fick et al. 2010, Escher, Baumgartner et al. 2011), and evidence suggests they are not readily removed from the waste stream (Kahle, Buerge et al. 2008, Peng, Huang et al. 2012, Assress, Selvarajan et al. 2019, Assress, Nyoni et al. 2020, Assress, Selvarajan et al. 2021), which could result in contamination of receiving environments (e.g., rivers, coastal waters, landspreading of sludge) (Kahle, Buerge et al. 2008, Martin and Hart 2023). In addition, approximately nine times more antifungals by mass are used in agricultural settings, as plant protection products, than in the clinic or veterinary settings (Fisher, Hawkins et al. 2018, Stevenson, Gaze et al. 2022). Whilst the concentrations used in agriculture are often lower than those used in the clinic (Gisi 2014), they are used much more frequently and are applied directly to land (Lago, Aguiar et al. 2014, Stevenson, Gaze et al. 2022), resulting in measurable concentrations in agricultural soils (Silva, Mol et al. 2019), and nearby waterways, following land runoff (Navarro, de la Torre et al. 2024). Several clinical and agricultural antifungals were included on the European Commission's Water Framework Directive Watch List in the 2020, 2022 and 2025 iterations (European Commission 2020, European Commission 2022, European Commission 2025). Substances are included on the Watch List as a result of their perceived risk to the aquatic environment, where more data is required to assess their actual risk (European Commission 2015). At the time of writing, European regulations do not include 'selection for resistance' as part of their environmental risk assessment (Agerstrand, Berg et al. 2015, European Medicines Agency 2024). In part, this is because there is no agreed-upon approach on how this should be carried out; yet this is likely critical to mitigating the risk of the selection and dissemination of antifungal resistance (AFR) in the environment.

To date, research determining the lowest concentration at which selection for resistance can occur has been biased towards antibiotics and water environments for both modelled (Bengtsson-Palme and Larsson 2016, Rico, Jacobs et al. 2017, Zhang, Ge et al. 2022) and experimental approaches (Lundstrom, Ostman et al. 2016, Kraupner, Ebmeyer et al. 2018, Murray, Zhang et al. 2018, Kraupner, Ebmeyer et al. 2020, Murray, Stanton et al. 2020,

Stanton, Murray et al. 2020), with a distinct lack of work establishing safe discharge limits for antifungals and for other environmental matrices (Murray, Stanton et al. 2024). The lack of data for, and experimental tools to determine antifungal selective concentrations have previously been highlighted (Environment Agency 2022, Stevenson, Gaze et al. 2022). In addition, a semi-systematic search of the literature identified selective concentrations for all antimicrobials found that, of 331 selective endpoints defined for all antimicrobials (Environment Agency 2024, Murray, Stanton et al. 2024), only five were determined for antifungal compounds, all of which were all calculated in one study (Bengtsson-Palme and Larsson 2016). This one study predicted no effect concentration for the selection of resistance (PNECRs) from minimum inhibitory concentrations (MICs) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) database, resulting in antifungal PNECRs ranging from 0.008 (itraconazole) to 0.25 µg/L (fluconazole) (Bengtsson-Palme and Larsson 2016). However, this approach, and subsequent modelling studies (Rico, Jacobs et al. 2017, Menz, Olsson et al. 2019, Zhang, Ge et al. 2022, Environment Agency 2024), have not accounted for uncertainty arising from the censored nature of experimental observations of MICs.

To address the limited data on PNECRs for antifungals, as well as the lack of models to determine selective concentrations that consider data censoring, this study aimed to:

1. Generate PNECR values for water environments ($PNECR_{water}$) by fitting Species Sensitivity Distributions (SSDs) using a Maximum Likelihood Estimation (MLE) approach to estimate the lower 5^{th} percentile Hazard Concentrations (HC5s) from censored species/compound level MIC data, and applying an assessment factor to generate a $PNECR_{water}$.

2. Generate PNECR values for antifungals in a “model” soil environment ($PNECR_{soil}$) using the equilibrium partitioning method to extrapolate the selective potential of bioavailable antifungal concentrations in bulk soil (presuming the only bioavailable fraction is that in the pore water) from the $PNECR_{water}$.

3. Generate risk quotients (RQs) by comparing the $PNECR_{water}$ and $PNECR_{soil}$ generated to measured environmental concentrations (MECs) to understand the potential risk current environmental concentrations of antifungals pose.

Materials and Methods

Figure 1 highlights a flow diagram for the methodology used in this study, with details on each of these sections described below.

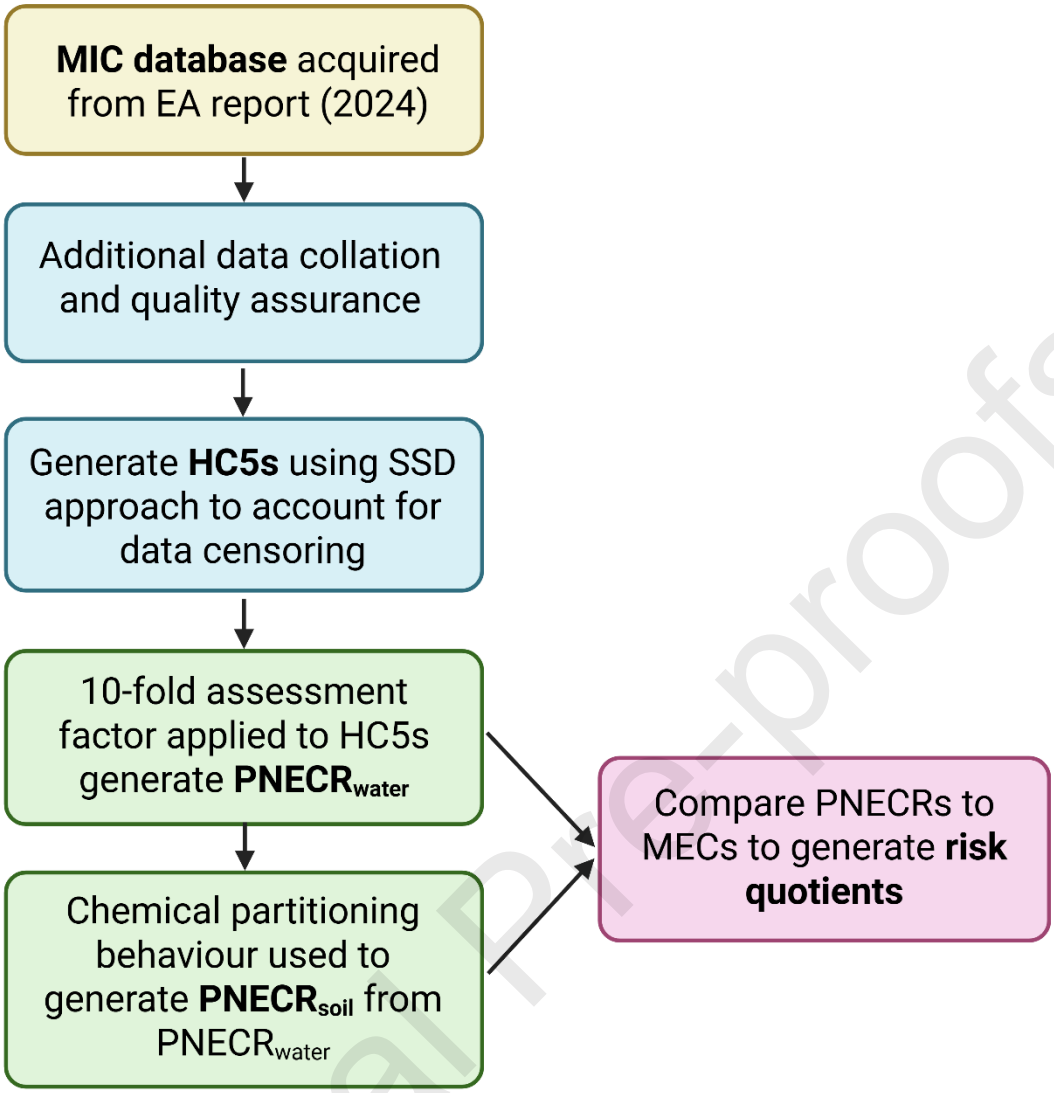


Figure 1. Flow diagram showing the overview of the methodology undertaken in this study. EA report refers to a report titled "Determining selective concentrations for antibiotics and antifungals in natural environments" by the Environment Agency (Environment Agency 2024). HC5s = lower 5th percentile Hazard Concentrations. $PNECR_{water}$ = predicted no effect concentration for resistance in water. $PNECR_{soil}$ = predicted no effect concentration for resistance in water. MECs = measured environmental concentrations. (Created with Biorender).

MIC database

MIC data for this study was obtained from an Environment Agency report, which was a precursor to the work presented here (hence forth referred to as "the EA report") and used a different approach, which did not take into account the censored nature of MIC data, to determine $PNECRs_{water}$ (Environment Agency 2024). In the EA report, MIC data for 18 clinical and 8 agricultural antifungals was collated, totalling 401,586 data points. For the present study, the publications and datasets included the EA report's MIC database were searched for extra data required for the methodology used here (the specified antifungal concentrations tested). Further, an additional quality assurance step was undertaken, as required by the new methodology: if the range of concentrations tested was not specified in either the methods or

results, the dataset was excluded. This was because the highest and lowest test concentrations were unknown, making it impossible to determine the concentrations range within which reported MIC values lay. Undertaking these additional steps reduced the number of studies and isolates in the database. However, where isolates were included, there was high confidence about the MIC being used (i.e., there were known upper and lower boundaries for the MIC).

Metadata analyses

Metadata analyses were conducted on extracted isolate data in the MIC dataset after quality assessment. Metadata analyses were grouped into the topic areas: antifungal type; testing methodology; and fungal taxa. Data is presented as percentages of the "total isolates" collated for the purpose of analysing MIC data. However, it should be noted that some of the same fungal isolates have been tested against multiple antifungals for multiple antifungals in the literature; therefore, the actual number of individual fungal isolates tested will be lower.

Generating $PNECRs_{water}$

The majority of the MIC data is interval censored, with experiments identifying a specific range of values within which the true MIC lies. For example, a study designed to test isolates in successive two-fold dilutions can only determine that the MIC of a compound lies between the highest concentration at which inhibition was not detected and the lowest concentration at which inhibition was first detected. In this case, it can only be concluded that the true MIC lies between these two concentrations and not that either of these values is the MIC. Some MIC data is left censored; for these, the MIC was determined to lie below a specified value, typically the lowest concentration tested in the experiment. Similarly, for MIC values that were right censored, it was determined that the true MIC lay above a certain value, typically the maximum concentration tested, but again, the true value could not be determined. Previous methods generating predictions of PNECRs for antibiotics have not considered interval censoring (Bengtsson-Palme and Larsson 2016, Rico, Jacobs et al. 2017, Menz, Olsson et al. 2019, Zhang, Ge et al. 2022, Environment Agency 2024), thereby increasing the uncertainty of PNECRs generated from such data. MLE approaches can statistically account for data censoring and rigorously consider the effect of variable data quality on quantities of interest, such as the HC5.

To generate $PNECRs_{water}$, we used a MLE approach to fit Species Sensitivity Distributions (SSDs) to estimate the HC5 for each compound, which aims to protect 95% of the species. Compounds for which fewer than 10 different fungal species were tested were excluded from PNECR determination as used in other published (Wheeler, Grist et al. 2002) and regulatory approaches (Whitehouse, Brown et al. 2011). For each remaining compound, we identified the minimum reported MIC (minMIC) for each species, and used this to construct MLE estimates of the SSD considering the log-normal, Weibull, and log-logistic models as our candidate distributions using the R-package fitdistrplus version 1.2 (Delignette-Muller and Dutang 2015) in R version 4.5.0 (R Core Team 2025). The Akaike Information Criterion was used for model selection (Sakamoto, Ishiguro et al. 1986), and model goodness-of-fit was assessed using diagnostic plots. A 95% confidence interval for the HC5 was computed using a non-parametric bootstrapping approach. Following this, an assessment factor of 10 was applied to derive the $PNECRs_{water}$, as has been previously used in similar studies to account for the difference between MIC and MSC (Bengtsson-Palme and Larsson 2016, Murray, Stanton et al. 2021), and is recommended as the assessment factor used in surface waters by the European Medicines Agency 2024 guidance on conducting environmental risk assessments for human medicines (European Medicines Agency 2024).

To assess the effect of data censoring on the HC5 prediction, we repeated the above procedure assuming, as previous studies have done (Bengtsson-Palme and Larsson 2016,

Rico, Jacobs et al. 2017, Menz, Olsson et al. 2019, Zhang, Ge et al. 2022), that the right limit of each censored interval was instead the true value of the experimentally observed MIC rather than the upper limit of the concentration at which inhibition was observed. For each compound, an SSD was fitted on the uncensored dataset, and the HC5 predicted by the model that did not account for data-censoring was compared to the HC5 of the model that incorporated data censoring.

Generating $PNECRs_{soil}$

To generate $PNECRs_{soil}$, partitioning coefficients were used to estimate the bulk soil concentration of antifungal needed to select for resistance within the pore water, assuming that only the pore water concentration was the only bioavailable fraction, as previous studies have presumed (Menz, Olsson et al. 2019, Elder, O'Neill et al. 2023). $PNECRs_{water}$ were converted to $PNECRs_{soil}$ using a series of equations adapted from European Chemicals Agency reports (European Chemicals Agency 2008) using bespoke Excel spreadsheets which can be found in Supplementary file 3.

Equation 1: Air-water partition coefficient (K_{aw}) (ECHA equation R.16-5) (European Chemicals Agency 2008)

$$K_{aw} = H \,/\, (R \times T)$$

Where:

K_{aw}	Air-water partition coefficient (dimensionless)
H	Henry's Law Constant in $Pa.m^3.mol^{-1}$ [chemical specific at 298K]
T	Temperature at the air-water interface in K [285 K or 12°C]
R	Universal Gas Constant in $Pa.m^3.mol^{-1}.K^{-1}$ [8.314]

Equation 2: Sorbed soil-water partition coefficient (K_d) (ECHA Equation R.16-6) (European Chemicals Agency 2008)

$$K_d = f_{oc} \times K_{oc}$$

Where:

K_d	Sorbed soil-water partition coefficient in L.kg
f_{oc}	Fraction of soil organic carbon in $kg.kg^{-1}$ [0.02 or 3.4% by wt. soil organic matter]

K_{oc} Organic carbon – water partition coefficient in L.kg [chemical specific]

212

213 *Equation 3: Total soil-water partition coefficient (K_{sw}) (ECHA Equation R.16-7) (European*
214 *Chemicals Agency 2008)*

215 $$K_{sw} = \theta_w + (K_{aw} \times \theta_a) + (\theta_s \times (K_d / 1000) \times \rho_s)$$
216

Where:

K_{sw} Total soil-water partition coefficient in $m^3.m^{-3}$

K_d Sorbed soil-water partition coefficient in L.kg

K_{aw} Air-water partition coefficient (dimensionless)

Θ_a Fraction of air-filled soil porosity in $m^3.m^{-3}$ [0.2]

Θ_s Fraction of water-filled soil porosity in $m^3.m^{-3}$ [0.6]

Θ_w Fraction of water-filled soil porosity in $m^3.m^{-3}$ [0.2]

ρ_s Solid phase density in $kg.m^{-3}$ [2500]

217

218 *Equation 4: Total soil concentration at porewater concentration equal to PNECR for water*
219 *(Rearranged ECHA Equation R.16-55) (European Chemicals Agency 2008)*

220 $$PNECR_{soil} = (K_{sw} \times PNECR_{water} \times 1000) / \rho_{bd}$$

Where:

$PNECR_{soil}$ Predicted no effect concentration for resistance (PNECR) in $mg.kg^{-1}$ DW

K_{sw} Total soil-water partition coefficient in $m^3.m^{-3}$

$PNECR_{water}$ Predicted no effect concentration for resistance (PNECR) from water in mg/L [chemical specific]

ρ_{bd} Dry bulk density of soil in $kg.m^{-3}$ [1300]

This method utilised modelled physico-chemical properties obtained from EPI Suite version 4.1(US EPA 2024). Koc values were estimated from logKow originating from the KOCWIN programme. Only EPI Suite values were used to ensure standardisation, however, empirical values were collated and are presented in Supplementary file 1 and can be used in editable Excel sheets presented in Supplementary file 3. This allows for readers to conduct these calculations so that $PNECRs_{soil}$ can be determined for specific soil types.

Collating MECs and generating risk quotients (RQs)

Collating MECs

MECs for natural and wastewater environments were identified using the Umweltbundesamt (UBA) “Pharmaceuticals in the environment” database (Umweltbundesamt 2022) which collates global data on the reported levels of pharmaceuticals in a range of environments. These were filtered to include wastewater influent and effluent, surface water, groundwater and soil.

Generating risk quotients (RQs)

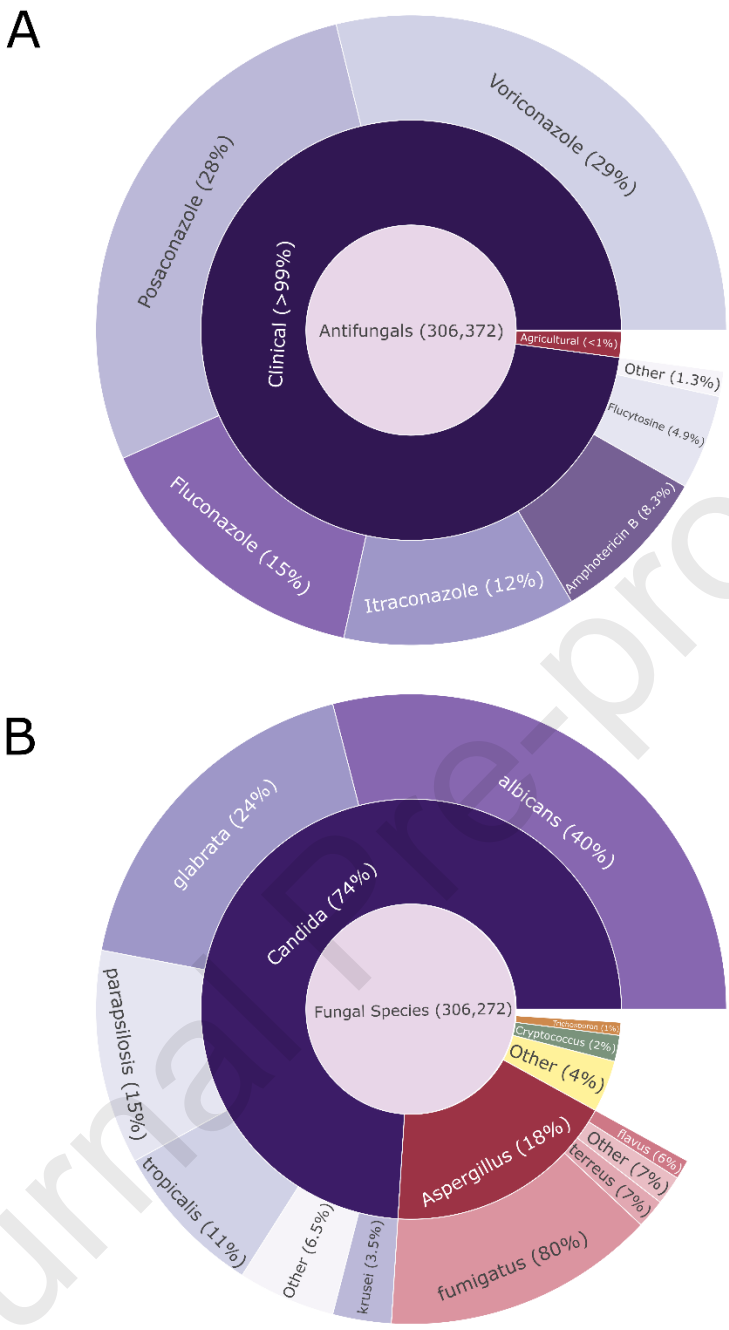
Risk quotients (RQs) are generated by dividing MECs by the PNECR, which allows for the MEC risk to be determined (European Medicines Agency 2024). An RQ ≥ 1 indicates a potential risk of selection in that specific environment.

Results

MIC fungal database and associated metadata

Following the enhanced quality assessment on the MIC database obtained from the EA report, 106 publications and two grey literature databases provided MIC values for 306,372 isolates for 24 antifungals (clinical antifungals = 18, agricultural antifungals = 6). This data, along with associated metadata (antifungal, reported MIC value and unit, standardised MIC value, fungal species, susceptibility interpretation, testing methodology and reference) can be found in Supplementary File 1 – MIC database. Notably, in the majority of studies, the same fungal isolates were tested on multiple antifungals. Therefore, the phrase “number of isolates” used throughout denotes all data collected, including where the same fungal isolate has been tested against multiple antifungals.

Analysis of the metadata after quality assessment showed a bias towards clinical antifungals (99.96% clinical vs. 0.04% agricultural) (Figure 2A) as well as a few key fungal species of clinical relevance (74.23% *Candida* spp. and 18.37% *Aspergillus* spp.) (Figure 2B). In addition, the majority of MICs were derived using one of two standardised approaches (CLSI or EUCAST, 69.28% and 28.71%, respectively), suggesting that much of the data was collected using repeatable and robust methodologies.



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257 **Figure 2A. Proportion of MIC values by antifungal type, grouped by number of isolates.**
258 **Figure 2B. Proportion of MIC values by fungal genera and species, grouped by number**
259 **of isolates.** Fungal isolate number (centre of figures), indicated the total number of MIC data
260 points in the database. Percentage values represent the percentage of the total number of
261 MIC data points. However, it should be noted that some of the same fungal isolates have MIC
262 data presented for multiple antifungals in the literature; therefore, the actual number of
263 individual fungal isolates tested will be lower.

264

Further, Table 1 highlights the range and median of MICs found for observed data (i.e., data that was not censored) for each antifungal after quality assessment, as well as the number of MIC values identified with left and right censored data.

Table 1. Summary of antifungal MICs and metadata. MIC = minimum inhibitory concentration. “Total number of species” has names standardised as described in the method section above. “Observable” data = data that was not left or right censored. N/A = not applicable – all data for bifonazole was left censored and therefore, no observable data, meaning no range or median MIC values were able to be presented here.

Antifungal	Total number of isolates	Total number of species	MIC range (µg/ml)	MIC median of observable data (µg/ml)	Number of isolates with left censored data	Number of isolates with right censored data
Amorolfine	412	16	0.01 - 64	0.04	4	6
Amphotericin B	25293	121	0.003 - 128	0.25	46	38
Azoxystrobin	7	7	0.3125 - 10	2.5	0	0
Bifonazole	14	1	N/A	N/A	14	0
Clotrimazole	558	23	0.003 - 40	0.5	66	0
Cyproconazole	20	1	64 - 128	64	0	0
Difenoconazole	50	9	0.3125 - 8	2.5	0	41
Econazole	73	5	0.25 - 8	1	0	0

Enilconazole	78	2	0.016 - 2	0.06	0	0
Fluconazole	47329	92	0.03 - 256	1	311	537
Flucytosine	14952	46	0.008 - 256	0.25	5444	147
Griseofulvin	406	10	0.016 - 64	1	0	0
Itraconazole	37866	120	0.004 - 125	0.25	304	660
Ketoconazole	588	35	0.003 - 32	0.5	38	23
Miconazole	394	17	0.015 - 64	0.5	6	0
Nystatin	253	13	0.25 - 320	2	0	0
Posaconazole	87123	111	0.007 - 64	0.06	749	494
Prochloraz	3	3	0.5 - 2	1	0	0
Tebuconazole	43	2	2 - 32	16	0	33
Terbinafine	1094	41	0.002 - 256	0.25	115	133

Tioconazole	105	9	0.016 - 16	4	0	0
Tolnaftate	128	1	0.016 – 0.5	0.016	0	0
Trifloxystrobin	7	7	0.625 - 10	2.5	0	0
Voriconazole	89576	114	0.002 - 512	0.06	1269	375

$PNECRs_{water}$

MICs were available for 24 of the 53 antifungals of interest to this study. $PNECRs_{water}$ were generated for 13 of these 24 antifungals (Table 2), with the remaining 11 excluded because the MIC data was from fewer than 10 distinct fungal species. The 13 antifungals for which $PNECRs_{water}$ could be calculated were all clinical antifungals (Table 2). The high degree of uncertainty observed in some of the estimates of HC5 (for example, clotrimazole) can be attributed to a high degree of left censoring in the underlying data (around 39% for clotrimazole, see Methods for an explanation of data censoring). This indicates that the most sensitive isolates of the species tested routinely had MICs below the lowest test concentration, resulting in low HC5s with large uncertainties as a result of the majority of the available data representing non-detects. To demonstrate the effect that censored data had on our predictions we repeated the analysis under the assumption that the underlying MIC data were not censored and were, instead, direct observations of true MICs. This resulted in predictions of HC5 that were higher in the case of every compound, and which were often substantially more certain than the experimental observations support (see Supplementary File 2). This highlights the importance of accounting for the methodological uncertainty inherent in experimental observations of MICs.

Table 2. $PNECRs_{water}$. The "Number of fungal species tested" column indicated for each antifungal is the number of unique fungal species for which the MIC was experimentally determined. HC5 = lower 5th percentile Hazard Concentrations.

Compound	HC5 (95% CI) (µg/L)	Number of fungal species tested	$PNECR_{water}$ (µg/L)
Amorolfine	1.34 (0.278 - 10.2)	16	0.134 (0.0278 - 1.02)
Amphotericin B	0.873 (0.274 - 2.52)	121	0.0873 (0.0274 - 0.252)
Clotrimazole	0.00567 ($7.37x10^{-06}$ - 0.569)	23	0.000567 ($7.37x10^{-07}$ - 0.0569)
Fluconazole	9.90 (4.07 - 23.5)	92	0.990 (0.407 – 2.35)
Flucytosine	0.445 ($1.51x10^{-02}$ - 4.92)	46	0.0445 ($1.51x10^{-03}$ – 0.492)

Griseofulvin	1.12 (3.52x10^{-03} - 19.7)	10	0.112 (3.52x10^{-04} – 1.97)
Itraconazole	0.151 (3.92x10^{-02} - 0.472)	120	0.0151 (3.92x10^{-03} - 0.0472)
Ketoconazole	0.0154 (1.91x10^{-04} - 0.383)	35	0.00154 (1.91x10^{-05} - 0.0383)
Miconazole	0.370 (7.77x10^{-04} - 17.3)	17	0.0370 (7.77x10^{-05} – 1.73)
Nystatin	79.4 (41.9 - 287)	13	7.94 (4.19 – 28.7)
Posaconazole	0.771 (0.287 - 1.97)	111	0.0771 (0.0287 – 0.197)
Terbinafine	0.478 (2.63x10^{-03} - 4.87)	41	0.0478 (2.63x10^{-04} – 0.487)
Voriconazole	0.115 (2.69x10^{-03} - 0.457)	114	0.0115 (2.69x10^{-04} - 0.0457)

Although 11 of the antifungals with MICs did not meet the data requirements for $PNECR_{water}$ calculation, we have presented the lowest MIC values found in the literature for each of them for reference (Supplementary file 2 – Table 1).

$PNECRs_{soil}$

The $PNECRs_{soil}$ can be found in Table 3, which should be interpreted as being the concentration of the antifungal in a 'model' soil that would be sufficient to allow for the pore water concentration of antifungal to equal that of the $PNECR_{water}$. Soil type specific physico-

chemical properties, derived empirically, can be found in Supplementary file 1 and bespoke Excel spreadsheets that were used to calculate these $PNECRs_{soil}$ can be found in Supplementary file 3. These spreadsheets can be amended with the soil type specific physico-chemical properties in Supplementary file 1 for soil type specific $PNECRs_{soil}$.

Table 3. $PNECRs_{soil}$. * = chemical properties were estimated based on the SMILES in EPI Suite, not based on CAS numbers.

Antifungal	$PNECR_{soil}$ (mg/kg)
Amorolfine*	0.0379 (0.00786 – 0.288)
Amphotericin B	$1.34x10^{-5}$ ($4.22x10^{-6}$ – $3.88x10^{-5}$)
Clotrimazole	0.000800 ($1.04x10^{-6}$ – 0.0803)
Fluconazole	0.000319 (0.000131 – 0.000757)
Flucytosine	$8.14x10^{-6}$ ($2.76x10^{-7}$ – $9.00x10^{-5}$)
Griseofulvin	0.000920 ($2.89x10^{-6}$ – 0.0162)
Itraconazole	0.230 (0.0597 – 0.719)
Ketoconazole	0.000111 ($1.37x10^{-6}$ – 0.00275)
Miconazole	0.0583 (0.000122 – 2.73)

Nystatin	2.16 (1.14 – 7.82)
Posaconazole	0.00182 (0.000676 – 0.00464)
Terbinafine	0.0166 ($9.16x10^{-5}$ – 0.170)
Voriconazole*	$2.26x10^{-6}$ ($5.29x10^{-8}$ – $8.89x10^{-6}$)

RQs based on generated PNECRs

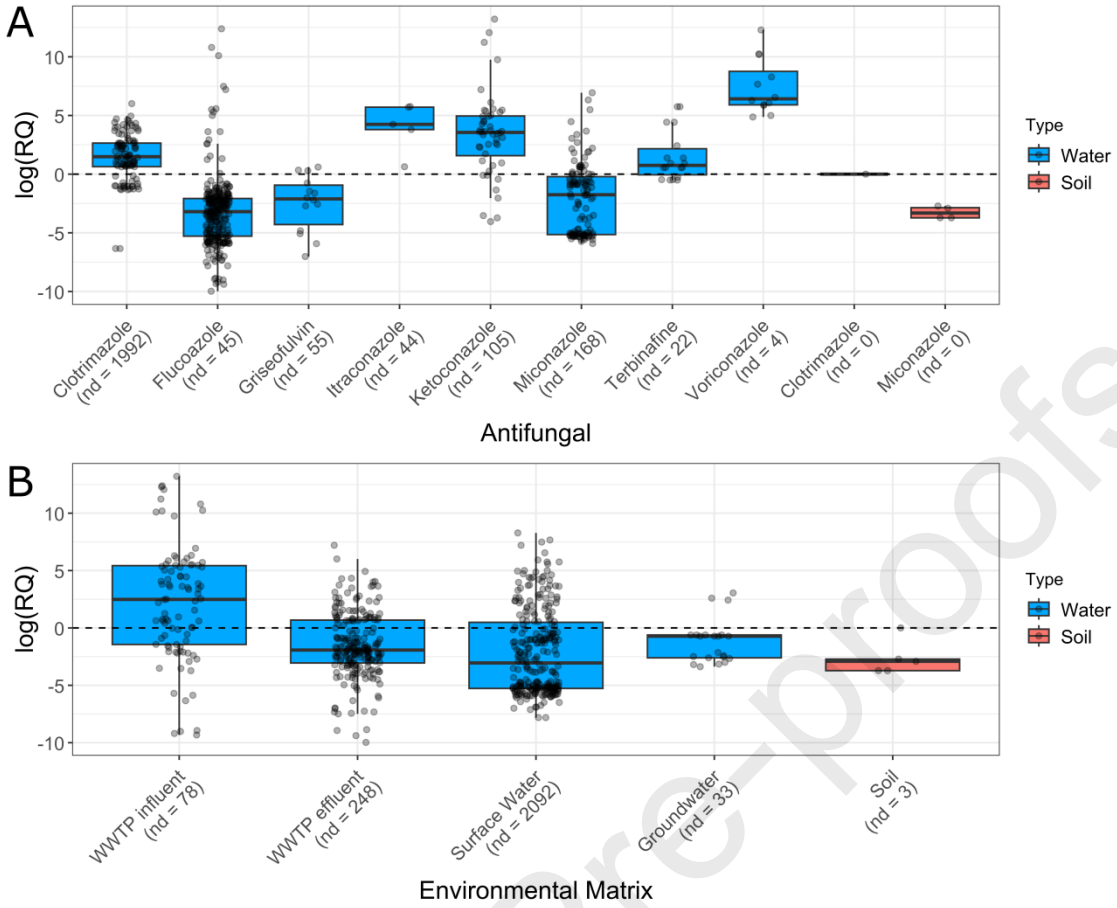
RQs generated from MECs in water environments for all antifungals, 6.54% (n = 200) of these were above 1 (indicating a risk of selection). Two antifungals were never found in the environment at concentrations that resulted in an RQ exceeding 1 (nystatin and posaconazole); whereas voriconazole exceeded an RQ of 1 in 75% (n = 12) of the water samples in which it was measured. For soil environments, 12.5% (n = 1) of RQs were above 1. Fluconazole, itraconazole, ketoconazole and miconazole were never found in the soil environment at concentrations that resulted in RQ exceeding 1; whereas in all soil samples in which clotrimazole was measured, it was at concentrations that would be expected to select for resistance based on the PNECR (i.e., RQ ≥ 1). It is of note that data availability was far greater for MECs in water environments (n = 3059) in comparison to soil (n = 8) environments, which limits the risk assessment able to be undertaken in soils. A detailed summary of key findings can be found in Table 4.

Table 4. Summary of MEC data, from the UBA database (Umweltbundesamt 2022), and the percentage of these that generate RQs greater than 1. MEC = measured environmental concentration, RQ = risk quotient. RQ greater than 1 indicates that there is a risk of AFR developing. * = percentage includes non-detect values.

Antifungal	Total number of reported MECs	Number of non-detects reported	Percentage of RQs ≥ 1 (%)*	Median RQ with non-detects	Median RQ without non-detects
Water					
Clotrimazole	2098	1992	3.86	0	4.42

Fluconazole	327	45	5.50	0.0244	0.0409
Griseofulvin	69	55	4.35	0	0.122
Itraconazole	49	44	10.2	0	69.5
Ketoconazole	153	105	26.1	0	35.0
Miconazole	291	168	9.62	0	0.173
Nystatin	2	2	0	0	N/A
Posaconazole	14	14	0	0	N/A
Terbinafine	40	22	32.5	0	2.14
Voriconazole	16	4	75	404.3	617.4
Soil					
Clotrimazole	1	0	100	1	1
Fluconazole	1	1	0	0	N/A
Itraconazole	1	1	0	0	N/A
Ketoconazole	1	1	0	0	N/A
Miconazole	4	0	0	0.0395	0.0395

A graphical overview of the RQ data, excluding the incidents where the antifungal was below the limit of detection, can be found in Figure 3A, split by antifungal, and Figure 3B, split by environmental matrices. The number of incidents reported in the UBA where the antifungal was not detected is reported in the x-axis label as “nd =”.



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331

Figure 3A. RQ values shown by antifungal, grouped into water and soil. Figure 3B. RQ values shown by environment subtype, grouped into water and soil. nd = number of measured environmental concentrations (MECs) below the limit of detection – these are not represented in the boxplots, RQ = risk quotient. Note that y-axis for both 2A and B show log(RQ) and not RQ. Log(RQ) = 0 is the equivalent of RQ = 1, representing a risk of selection for AMR occurring. This is indicated by the black dashed line. MECs were available for nystatin and posaconazole in water; and fluconazole, itraconazole and ketoconazole in soil, but as these are all below the limit of detection, these are not represented on either 2A or B. Other antifungals where PNECRs were calculated that are not shown in 2A did not have any MECs on the UBA database, so RQs could not be calculated. Box plot centre line represents the median; with box limits showing upper and lower quartiles. The whiskers show 1.5x interquartile range and the points show all data points including outliers.

332

333 # Discussion

334 ## Comparison to previous work

335 This study calculates $PNECRs_{water}$ using SSDs which are a widely used approach to assess
336 risk in ecotoxicological settings (Fox, van Dam et al. 2021)Although previous studies have
337 used SSDs to produce estimates of PNECRs for antibiotics (Rico, Jacobs et al. 2017, Menz,
338 Olsson et al. 2019, Zhang, Ge et al. 2022), the only work studies we are aware of that predicted

339 $PNECRs_{water}$ for antifungals both used a mechanistic approach (Bengtsson-Palme and
340 Larsson 2016, Environment Agency 2024). Statistical approaches that express confidence in
341 estimates of quantities, such as HC5, are invaluable tools for regulators due to the varying
342 amount and quality of the data available for different compounds. However, despite high levels
343 of data censoring being common in experimentally derived MICs, the effects of censoring are
344 often not incorporated into estimates of risk (Bengtsson-Palme and Larsson 2016, Rico,
345 Jacobs et al. 2017, Menz, Olsson et al. 2019, Zhang, Ge et al. 2022). This methodological
346 omission is significant as, in this study, for example, 28% of the 116 isolates identified as most
347 sensitive to voriconazole were left censored, meaning that the MIC of the most sensitive
348 isolate lay below the lowest concentration tested. By treating the lowest level of detection as
349 the true MIC value, we produced an estimate of HC5 that is around 27 times higher than the
350 underlying experimental data supports once accounting for data censoring (see
351 Supplementary file 2). Repeating this analysis for all modelled compounds, we find that
352 predictions made by the SSDs that do not consider data censoring are always higher than
353 those made by the models that do. Further, in 8 out of 13 cases, predictions are outside the
354 95% confidence interval of the censored estimate (see Supplementary File 2 for further
355 comparison). This demonstrates that by failing to account for the variable data quality inherent
356 in experimental MIC observations we risk systemically overestimating "safe" concentrations of
357 antifungals in water. Other commonly used approaches to deal with non-detects, such as
358 deletion and substitution, do not rigorously deal with the uncertainty associated with
359 measurements below the level of detection, therefore are not appropriate for highly censored
360 data (Helsel 2010).

361 In this study, $PNECRs_{water}$ were converted to $PNECRs_{soil}$ using the equilibrium partitioning
362 method to take bioavailability into account. Previous studies have investigated the selective
363 potential of antibiotics in soil or land amended with different types of manure (Menz, Olsson
364 et al. 2019, Elder, O'Neill et al. 2023). Instead of generating PNECRs values specific to soil,
365 both studies assumed that the concentration of the antibiotic in the pore water was the only
366 fraction that would select for resistance (Menz, Olsson et al. 2019, Elder, O'Neill et al. 2023).
367 By considering the partitioning of the antibiotic, both calculated the concentration of the
368 antibiotic in the pore water and, by assuming this was the only bioavailable component, they
369 compared this to $PNECRs_{water}$ to generate RQs. Further, both used soil-type-specific physico-
370 chemical values, which could easily replace the EPI Suite modelled values employed in this
371 study (Menz, Olsson et al. 2019, Elder, O'Neill et al. 2023).

372 The only peer-reviewed study that has calculated PNECRs for AFR is the Bengtsson-Palme
373 and Larsson (2016) publication. This paper's primary focus was antibiotics but included a small
374 subset of antifungals in the analysis (Bengtsson-Palme and Larsson 2016). $PNECRs_{water}$ were
375 calculated for three of the same antifungals in both this study and the previous study. In this
376 study, $PNECRs_{water}$ were approximately two to five times higher than those derived in the
377 previous study (Bengtsson-Palme and Larsson 2016). However, whilst the $PNECRs_{water}$
378 calculated here were based on the HC5, the previous study generated their $PNECRs_{water}$ by
379 using HC1 (the lower 1st percentile Hazard Concentrations) (Bengtsson-Palme and Larsson
380 2016). Computing HC1s using the methodology defined in this study, PNECRs lie between
381 one to three orders of magnitude lower than those produced using the HC5 and within one
382 order of magnitude of the Bengtsson-Palme and Larsson (2016) estimates (see
383 Supplementary file 2). Other reasons for the differences seen between $PNECRs_{water}$ between
384 the studies (Bengtsson-Palme and Larsson 2016) is that the EUCAST database (European
385 Committee on Antimicrobial Susceptibility Testing 2023), which was exclusively used to
386 generate the previous $PNECRs_{water}$ (Bengtsson-Palme and Larsson 2016) is limited to clinical
387 pathogens. In this study, MIC values were collated from fungal isolates on the EUCAST and
388 CDC databases, which include clinical pathogens, and from 106 academic publications,
389 encompassing a wider range of species, although pathogens and opportunistic pathogens
390 were overrepresented. This study also uniquely takes into account data censoring, whereas
391 the previous study (Bengtsson-Palme and Larsson 2016), and others in this field (Rico, Jacobs

et al. 2017, Menz, Olsson et al. 2019, Zhang, Ge et al. 2022), do not. Therefore, as a result of the larger number of data points, a wider range of species, and an enhanced methodology, our updated dataset may provide more environmentally realistic and statistically robust PNECRs. Regularly reassessing existing target $PNECRs_{water}$ as the body of MIC data increases, and as methodologies for predicting risk are refined, is an essential step in ensuring that regulatory targets remain informed

The MIC data used in this study was obtained from the EA report (Environment Agency 2024), but additional data was obtained from publications and datasets, as well as more stringent quality assessment undertaken. Both measures were required for the enhanced model used here. However, this has resulted in a reduced number of studies and isolates being included in the MIC database used to derive $PNECRs_{water}$. This has enabled comparisons between the results of the methods applied in the EA report and this study, highlighting any impacts directly influenced by the enhanced methodology. In the case of the $PNECRs_{water}$, all of the PNECRs derived with the enhanced methodology here are lower than those derived in the EA report, which did not account for data censoring (Environment Agency 2024). This ranged from 5 to approximately 2,800 times lower, with a median difference of approximately 35 times lower. In addition, because of enhanced quality assessment of MIC data, four antifungals (econazole, enilconazole, tioconazole and tolnaftate) no longer satisfied the data requirements for the model in the present study. Similarly, with the soil samples, $PNECRs_{soil}$ were either the same value (itraconazole) or lower (between approximately 6 to approximately 2,375 times lower) in this study in comparison to the EA report (Environment Agency 2024). As with the $PNECRs_{water}$, the same four compounds did not have $PNECRs_{soil}$ generated for them, as there were no $PNECRs_{water}$ to convert into soil values. However, as a result of more soil property data being available on EPI Suite for a greater range of compounds since the EA report (Environment Agency 2024) was undertaken, PNECRs were able to be generated here for seven compounds (amorolfine, amphotericin B, fluconazole, flucytosine, griseofulvin, nystatin, voriconazole) that were not able to be generated in the report. For compounds where $PNECRs_{soil}$ were generated in both studies, differences in $PNECRs_{soil}$ values is likely to be as a result of considering the censored nature of MIC data to generate the $PNECRs_{water}$. This means that for both water and soil environments, the revised methodology presented here has resulted in PNECRs that are more conservative, and therefore, potentially more protective of AFR developing in these environments.

Environmental risk of antifungals

Generating PNECRs is important for assessing whether current MECs pose a risk for increasing resistance. Undertaking such assessments will identify environments where antifungal concentrations could increase the selection pressure, and subsequently AFR.

In general, wastewater influent had higher RQ values than wastewater effluent, surface water, groundwater, and soil. Whilst wastewater influent should be treated before being discharged into the natural environment, raw wastewater (which will consist of chemicals and microbes) can be released into downstream waterways via storm overflows during extreme weather (Tipper, Stanton et al. 2024). Whilst wastewater releases are seen as “point sources” of pollution, whereas land runoff is a “diffuse source”, therefore, the mitigation approaches used to target environments that may have RQs greater than 1 will differ depending on the source attribution of the antifungal. Land runoff may be more critical for agricultural antifungals, as they are directly applied to land through agricultural practices. However, the lack of data to inform PNECRs for agricultural antifungals prevent RQ determination.

Data limitations and future research priorities

The collated MIC data had biases, which resulted in the inability to calculate PNECRs for some important and widely used antifungals as there was insufficient data for the model. Further,

data was biased towards a limited range of clinical antifungals, with over 98.6% of MIC data derived for six clinical antifungals (voriconazole, posaconazole, fluconazole, itraconazole, amphotericin B, flucytosine), and biases towards two fungal species (i.e., *Candida* spp. and *Aspergillus* spp. comprised 92.6% of isolates). Limited MIC data for agricultural antifungals resulted in no PNECRs being calculated, which is a clear data and knowledge gap, as agricultural antifungals are applied directly into the environment as plant protection products (Stevenson, Gaze et al. 2022), and are used in significantly higher levels, by mass, than clinical or veterinary uses (Fisher, Hawkins et al. 2018, Stevenson, Gaze et al. 2022). Expanding the MIC data for both agricultural antifungals and the number of different fungal species will increase the number of PNECRs derived for other antifungals.

Whilst the MIC database consisted of over 300,000 values, there were a number of relevant publications where no raw data was publicly available. In addition, many studies (representing over 100,000 fungal isolates) were removed from the database as they did not meet the data requirements outlined in the Methods. Ensuring the availability of raw data and that methodology is sufficiently descriptive to interpret data would have substantially increased the number of MIC data points within the model. Even for compounds that met the inclusion criteria, predictions of HC5 (and consequently of PNECRs) are sometimes low and highly uncertain as a result of the high degree of left censoring present in the MIC data. In these cases, producing predictions of HC5 that are more certain, and therefore more likely to be protective, requires a combination of more data and the development of more sensitive experimental techniques that can directly observe MICs for compounds for which left censoring is regularly observed. For compounds where the MIC of fungal species is regularly found to be below the lowest antifungal concentration tested, more sophisticated statistical techniques, such as Bayesian Hierarchical models, could be used.

One point of uncertainty when calculating the $PNECR_{water}$ was the use of an assessment factor of 10. This number was based on previous work (Bengtsson-Palme and Larsson 2016, Murray, Stanton et al. 2021), as well as 2024 guidance for undertaking risk assessments of human medicines in surface water by the European Medicines Agency (European Medicines Agency 2024). Determining the most appropriate assessment factors is a growing research area with regards to bacteria and antibiotic resistance. For example, a recent study proposed choosing an assessment factor based on the fitness cost associated with harbouring plasmid-borne resistance (Kneis, de la Cruz Barron et al. 2025). However, understanding PNECRs for AFR, as well as the most appropriate assessment factor used to derive these, is in its infancy. As research in this area evolves, it may be found that 10 is not the most appropriate value to use. $PNECRs_{water}$ could be easily adjusted in the light of empirically-derived assessment factors, once these become available.

MEC data in the UBA database was often limited for antifungals, making it difficult to interpret the real-world context of the PNECRs generated. Further, there was a substantial difference between the number of MECs reported for antifungals of interest in this study in water environments (n = 3,059) and for those in soil (n = 8), representing a significant knowledge gap. Establishing more MEC data for all antifungals for a range of environmental matrices that have the potential to be polluted would increase understanding of where the highest risk is posed and identify whether and where mitigation measures are needed to prevent selection of AFR.

Method limitations and future research priorities

When calculating $PNECRs_{water}$, an underlying assumption of the SSD approach is that the species are representative of the broader fungal community. Given that *Candida* and *Aspergillus* spp. are overrepresented in the MIC dataset and the underrepresentation of other species known to be clinically, environmentally, and agriculturally important, this assumption may not be accurate with regards to actual risk of selection for all fungal species in a

community. Similarly, just under half of all compounds considered here were not tested against a sufficient number of unique species to robustly support the development of an SSD. In both cases, our estimates would be improved by further experimental work that aims to expand the range of species tested.

The equilibrium partitioning method used to convert $PNECRs_{water}$ to $PNECRs_{soil}$ is an established method used in ecotoxicology for non-resistance related PNECs (van Beelen, Verbruggen et al. 2003). This equation assumes that the only bioavailable component of the chemical exists in the pore water, and that the chemical sorbed to particulate matter is not bioavailable. It is unknown whether this is true for the selection of fungal resistance within soil communities. Further, the PNECRs are solely based on the parent antifungal, however, degradation products of an antifungal could retain some selective properties, as with other antimicrobials (Stanton, Murray et al. 2020). Hence, there is a need to examine the stable degradation products of antifungals and their behaviour in soils.

Conclusions

In this study $PNECRs_{water}$ and $PNECRs_{soil}$ were generated, and RQs were calculated to understand PNECRs in the context of the current measured environmental antifungal concentrations. Despite similar methodologies being used to generate PNECRs for antimicrobials previously (Bengtsson-Palme and Larsson 2016, Rico, Jacobs et al. 2017, Menz, Olsson et al. 2019, Zhang, Ge et al. 2022, Environment Agency 2024), this is the first time the censoring of MIC data has been taken into account when using this type of data to generate PNECRs. Following the development of this enhanced methodology, there is a difference between PNECRs generated here for three antifungals, in comparison to those previously generated (Bengtsson-Palme and Larsson 2016, Environment Agency 2024). This suggests that the re-evaluation of previously published antibiotic PNECRs, taking data censoring into account, is critical, particularly if these thresholds are used for environmental regulatory purposes.

Data Availability

All MIC, soil physico-chemical properties and MEC data collated and used during this work is provided in the Supplementary files. All code used to produce the $PNECRs_{water}$ results can be found here: https://github.com/DomBrass/AFR_SSDs. The equations used to produce the $PNECRs_{soil}$ can be found in bespoke spreadsheets in Supplementary file 3.

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Author contribution

ICS – conceptualisation, methodology, data curation, formal analysis, writing – original and draft, writing – review and editing, visualisation, supervision, project administration, funding acquisition. **DPB** – methodology, validation, formal analysis, visualisation, writing – original and draft, writing – review and editing. **HJT** – conceptualisation, methodology, data curation, formal analysis, writing – review and editing, funding acquisition. **RAP** – data curation, writing – review and editing. **AKM** – conceptualisation, methodology, validation, writing – review and editing, funding acquisition. **JMGS** – data curation, writing – review and editing. **AP** – methodology, visualisation, writing – review and editing. **DSR** – conceptualisation, writing – review and editing. **AH** – conceptualisation, supervision, funding acquisition, writing – review

and editing. **WHG** – conceptualisation, writing – review and editing, funding acquisition. **ACS** – conceptualisation, methodology, writing – review and editing, supervision, funding acquisition.

Competing interests

The authors declare no competing interests.

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Highlights

- Data censoring of MIC data was considered to determine PNECRs for antifungals.
- Over 300,000 MIC datapoints were collated, where clinical data was overrepresented.
- PNECRs were determined for 13 clinical antifungals for both water and soil.
- PNECRs were not determined for agricultural antifungals due to a lack of data.
- Risk quotients suggest current MECs could pose a risk for AFR development.

Author statement

ICS – conceptualisation, methodology, data curation, formal analysis, writing – original and draft, writing – review and editing, visualisation, supervision, project administration, funding acquisition. **DPB** – methodology, validation, formal analysis, visualisation, writing – original and draft, writing – review and editing. **HJT** – conceptualisation, methodology, data curation, formal analysis, writing – review and editing, funding acquisition. **RAP** – data curation, writing – review and editing. **AKM** – conceptualisation, methodology, validation, writing – review and editing, funding acquisition. **JMGS** – data curation, writing – review and editing. **AP** – methodology, visualisation, writing – review and

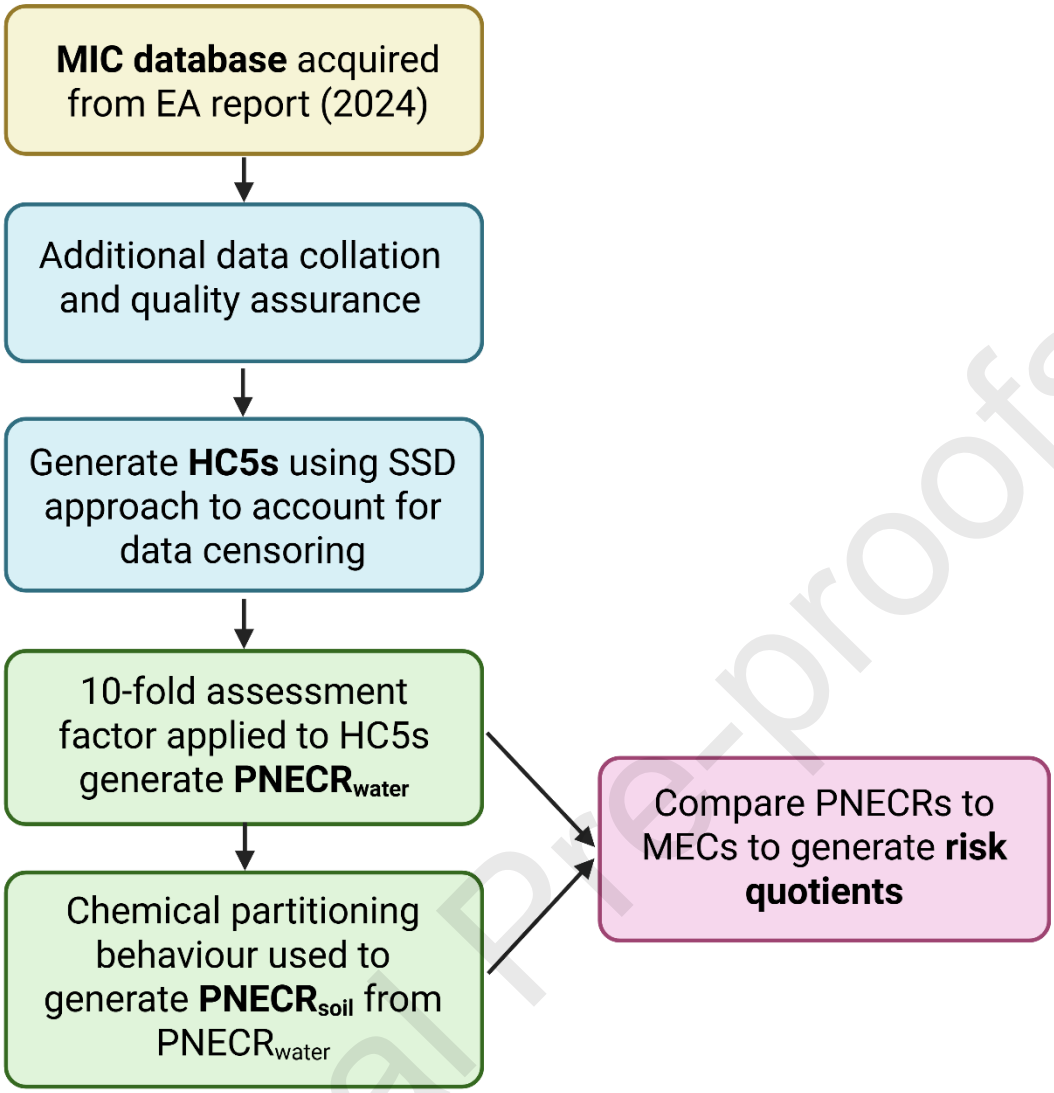
editing. **DSR** – conceptualisation, writing – review and editing. **AH** – conceptualisation, supervision, funding acquisition, writing – review and editing. **WHG** – conceptualisation, writing – review and editing, funding acquisition. **ACS** – conceptualisation, methodology, writing – review and editing, supervision, funding acquisition.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Isobel C Stanton reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Dominic P Brass reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Holly J Tipper reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Rachel A Payne reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Aimee K Murray reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Jennifer M G Shelton reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Adam M Pym reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Alwyn Hart reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Daniel S Read reports financial support was provided by UK Research and Innovation Natural Environment Research Council. William H Gaze reports financial support was provided by UK Research and Innovation Natural Environment Research Council. Andrew C Singer reports financial support was provided by UK Research and Innovation Natural Environment Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



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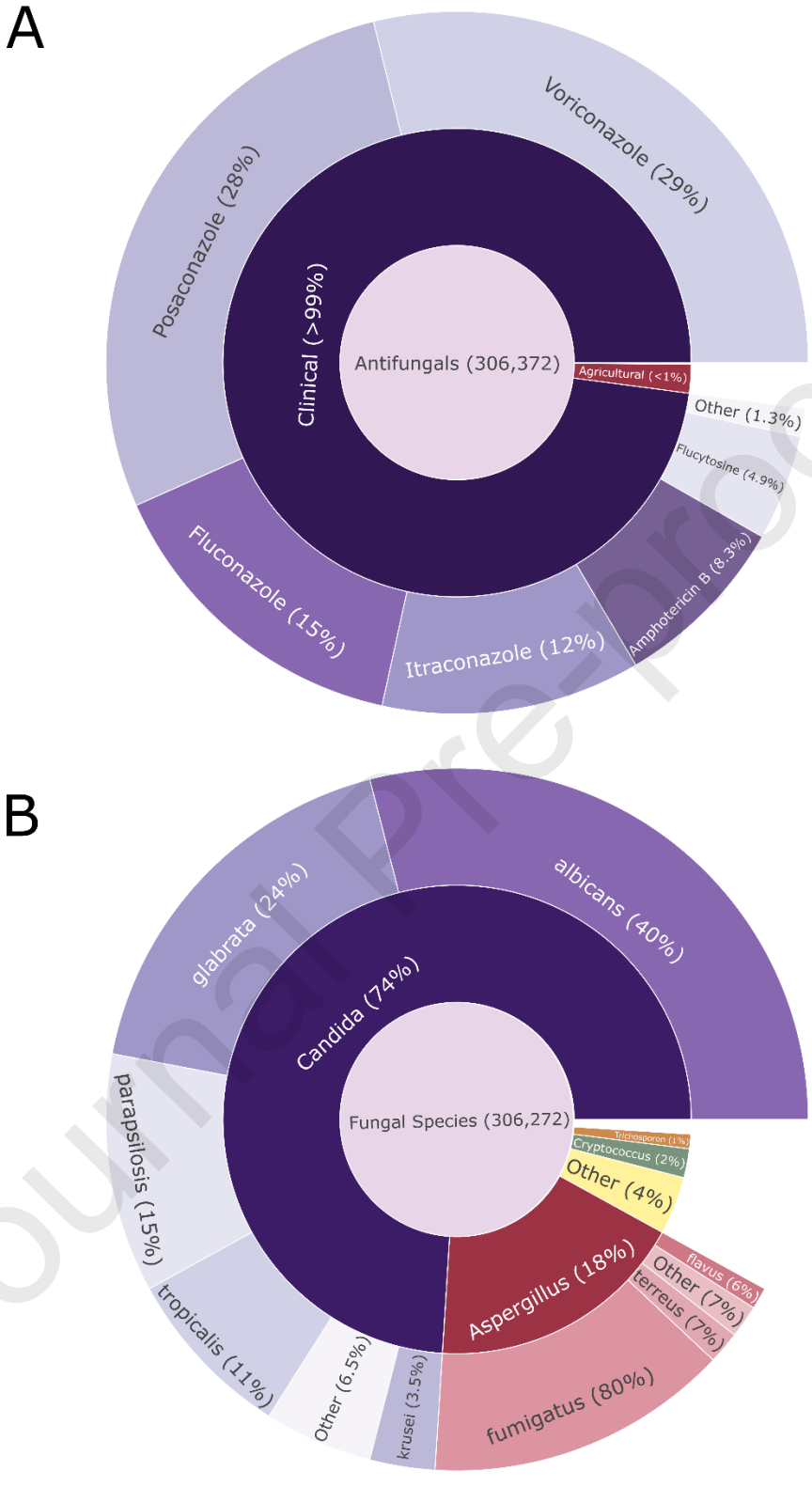

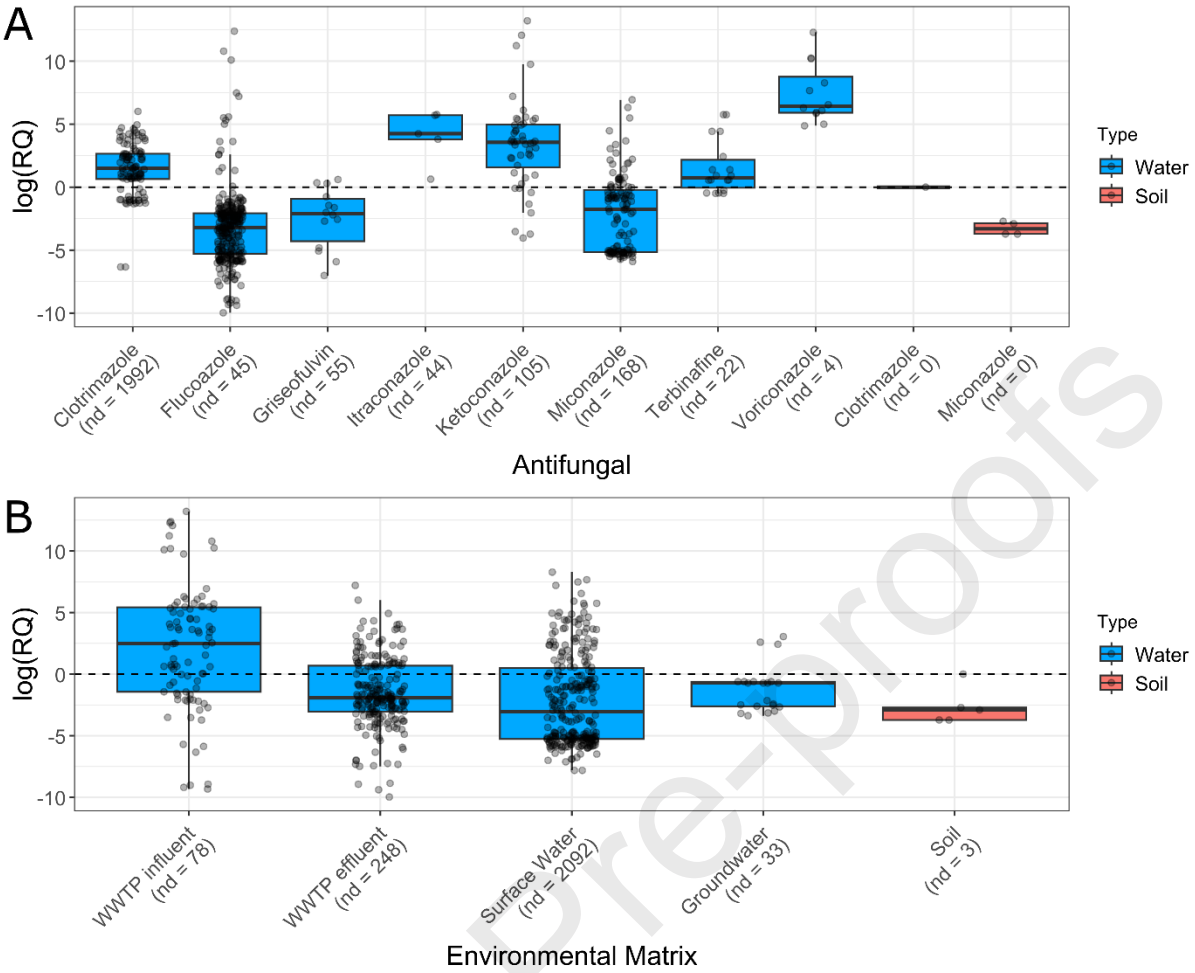

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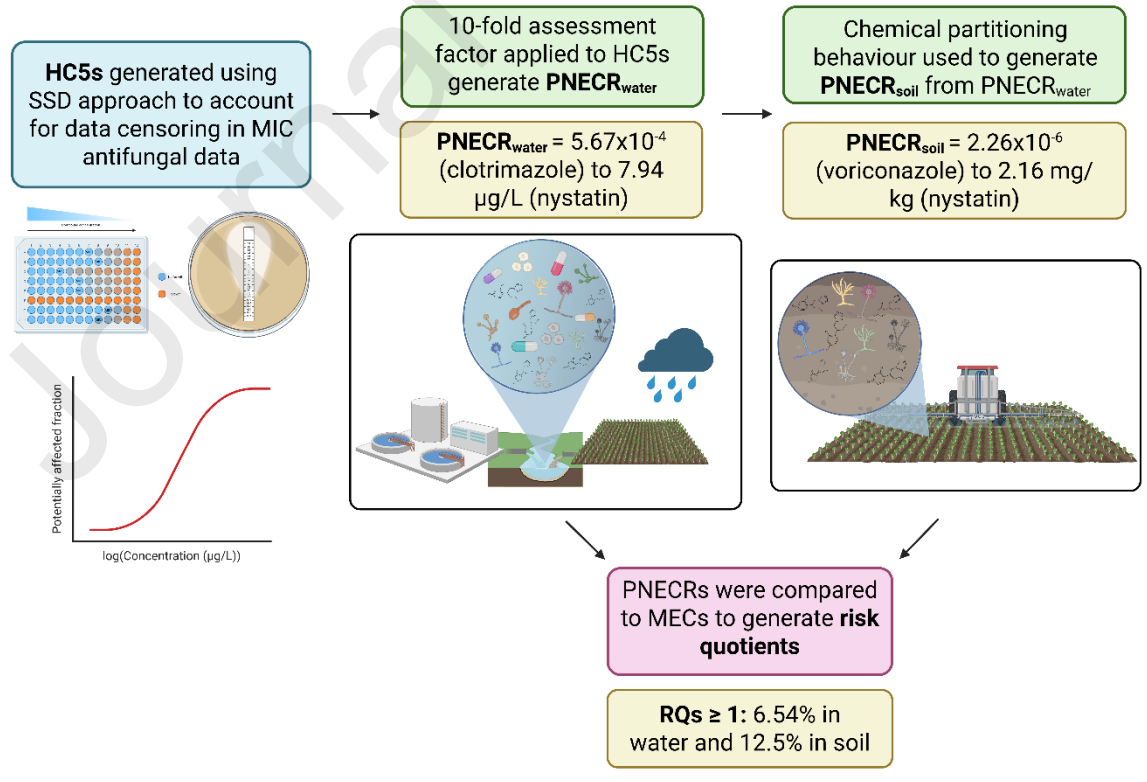

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