Scoping review into environmental selection for antifungal resistance and testing methodology

Can the novel SELECT method be modified to enable determination of selective concentrations for antifungal resistance?

July 2022

SC200011

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Published by:

Environment Agency
Horizon House, Deanery Road,
Bristol BS1 5AH

www.gov.uk/environment-agency

Further copies of this report are available from our publications catalogue: www.gov.uk/government/publications or our National Customer Contact Centre: 03708 506 506

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Keywords:
Antifungal resistance, antimicrobial resistance, evolution, selective endpoints, experimental evolution, environment

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Project number: SC200011

Citation: Environment Agency (2022) Scoping review into environmental selection for antifungal resistance and testing methodology. Environment Agency, Bristol.

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Executive summary

Antimicrobial resistance (‘AMR’) in pathogenic microorganisms is a significant global threat to human health. AMR within the clinic is well documented, though, additional settings are increasingly recognised to play a role in the evolution and spread of AMR. The environment, for example, harbours diverse reservoirs of AMR microorganisms and antimicrobial micropollutants, with most research to date focussing on antibiotic resistance in bacterial communities.

Fungal communities are complex - from single-celled yeasts to diverse multicellular moulds. Like bacteria and antibiotics, fungi have been documented to evolve resistance to antifungals following exposure to antifungal residues, in both the clinic and environment. Antifungal resistance (‘AFR’) is an emerging area of concern, with associated high mortality rates and few alternative treatment options available. However, environmental risk assessment (‘ERA’) guidelines e.g., for pesticides, do not require assessment of antifungal agents in terms of their ability to increase AMR, nor are there established experimental tools to measure this.

An important endpoint recently employed to determine selective concentrations of antimicrobials is the minimal selective concentration (‘MSC’). In this report, the MSC is defined as the lowest concentration of an antifungal at which positive selection for resistance occurs. There are currently no empirically determined MSC data available for antifungal agents. Thus, novel assays are required that can rapidly determine the MSCs of antifungals.

Various assays to determine MSC values for antibiotics have been designed and validated, though, many of these methods are costly and often laborious. Recently, a novel assay has been published that enables the rapid determination of antibiotic MSCs, using a significant reduction in growth of bacterial communities as a proxy for AMR: the ‘SELECT’ (SELection Endpoints in Communities of baCTeria) method (Murray et al., 2020). However, this method is currently optimised to measure antibiotic effects against communities of bacteria.

Therefore, this scoping document aimed to identify potential modifications to the SELECT method, to enable application and determine MSCs of antifungal compounds in fungi. Given that azole antifungals and fungicides are the only antifungal class deployed in both human/veterinary medicine and agriculture, this report will specifically focus on these compounds.

Through this work, it is reported that traditional ecotoxicologically derived endpoints may not be protective of AFR selection, highlighting the importance of providing experimental tools to empirically determine antifungal MSC data. Key considerations are noted, and preliminary modifications of the SELECT method are highlighted to enable the generation of antifungal MSCs using this method.

These include:

- The SELECT method may only provide an assay suitable for yeast species, not moulds.
- The use of haploid species *Candida auris* (*C. auris)* or *Nakaseomyces glabrata* (*N. glabrata,* previously *Candida glabrata*) for initial modifications is recommended, given that these species are of both environmental and clinical importance.
- For initial adaptations, instead of the original complex community inocula adopted for the SELECT method, an experimental inocula consisting of a mixture of resistant and susceptible strains of the same species is recommended. Reducing the complex community to a simple, single species culture allows greater control over experimental conditions and reduces the complexity of community interactions. However, fungi exist predominantly in complex communities in the environment, meaning environmental realism is reduced. Though, the mixing of susceptibility profiles provides greater real-world representation than a simple single species culture with similar susceptibility patterns.
- Adaptations of traditional antifungal MIC broth microdilution assays may apply as antifungal SELECT experimental conditions.

Validation assay suggestions are also provided, targeting documented AFR mechanisms. Both phenotypic and genotypic validation assays are noted, with advantages and disadvantages of such methods discussed and compared. In brief, phenotypic assays are beneficial as they generally encompass all resistance mechanisms responsible in a population. On the other hand, though genotypic assays usually focus on a single resistance determinant, they provide sensitive and accurate measures of resistance. The assays discussed target the two key mechanisms of AFR in fungi: overexpression or mutation of drug targets.

Novel experimental ideas are proposed in addition to considering whether the SELECT method may be adapted to test antifungals. These include the use of reduced ergosterol content as a proxy for AFR and a commonly applied invertebrate host model to determine if reduced virulence may also be used as a proxy for AFR. Importantly, these require additional testing before application.

To conclude, following the modifications outlined here, the SELECT method provides a promising tool to enable the rapid generation of selective endpoint concentrations for antifungals. Generation of MSC data is crucial for interpreting antifungal concentrations detected in the environment in terms of resistance selection risk, and such data can be considered in the development of thresholds of concern, which may be used to derive safe release limits or regulatory guidelines.

Based on this scoping review work, the project team published the ‘Hypothesis and Theory’ article “Antifungal Exposure and Resistance Development: Defining Minimal Selective Antifungal Concentrations and Testing Methodologies”.

1. Project aims

The role of the environment in the emergence of antifungal resistance ('AFR') has been previously highlighted (e.g., Jeanvoine et al., 2020; Monapathi et al., 2018), with particular concern owing to the direct application of effect concentrations of azole fungicides to agricultural crops and incomplete removal of pharmaceutical antifungals in wastewater treatment systems. Current environmental risk assessment ('ERA') guidelines, including for pesticides, do not require assessment of antifungals in terms of their ability to increase AFR. In addition, there are currently no universal assays that are able to empirically generate such data. Therefore, there is currently very little information available regarding the selective potential of antifungals at environmentally relevant concentrations.

The novel 'SELECT' (SELection Endpoints in Communities of baCTeria) method (Murray et al., 2020) offers a robust tool to routinely and inexpensively determine the lowest antibiotic concentration that selects for antimicrobial resistance ('AMR'), referred to as the minimal selective concentration ('MSC'). Though this method has only been used to test antibiotics against sewage-derived samples of bacteria, application to other antimicrobials and microorganisms may be achieved through assay modifications. Therefore, the primary aim of this report is to explore the options to adapt a SELECT-type approach for antifungal agents and fungi, in order to provide information on MSCs against which concentrations detected in the environment can be considered and interpreted.

To address this aim, this report will:

1. Present a brief literature review to provide background on:
    - AMR, AFR and the role of the environment in the evolution of AFR.
    - Current antifungal monitoring and risk assessment.
    - The SELECT method.
2. Summarise the information required for effective assessment of selection risk, including:
    - Key antifungal classes and their modes of action.
    - Occurrence, frequency and severity of key fungal infections, including *Candida* and *Aspergillus.*
    - Current antifungal selective pressure estimations.
    - Evidence supporting the link between antifungal use and resistance.
    - Key AFR determinants.
    - Fitness costs associated with AFR.
    - Environmental fate of antifungals.
    - Ecotoxicity testing.
3. Provide suggestions to adapt the existing SELECT method.
4. Suggest experimental validation assays.
5. Propose novel experimental alternatives.
6. Compare assays described.
7. Provide recommendations for future research.

2. Background

2.1. Antimicrobial resistance

Antimicrobials, including antibacterial, antiviral, antifungal and antiparasitic agents, sustain modern medical practice and are essential for biosecurity and protection of the global economy (Murray et al., 2018; O'Neil, 2015, 2014). However, the efficacy of antimicrobial therapy is increasingly challenged by the emergence and spread of AMR. AMR microorganisms, including bacteria, viruses, fungi and parasites, have the 'ability to multiply or persist in the presence of an increased level of an antimicrobial agent' (Ashbolt et al., 2013). It has been estimated that drug-resistant infections may result in 10 million human deaths annually by 2050 (O'Neill, 2014).

Applications of antimicrobials are broad and include human and/or veterinary medicine. In addition, antimicrobials are utilised as pesticides and biocides, including disinfectants in, for example, aquaculture, agriculture and horticulture (Hoelzer et al., 2017; Barnes et al., 2014; Jain et al., 2013; Andersson and Hughes, 2012; Verweij et al., 2009; Odds et al., 2003). Crucially, the evolution of resistance to these agents is not restricted to the clinic, with a growing body of research highlighting the importance of the environmental selection, evolution and dissemination of AMR (Murray et al., 2018). Owing to drug resistant infections of clinical importance, AMR is well represented in the literature, with respect to antibiotic resistance. In comparison, AFR is less studied and is the focus of this report.

2.2. Antifungal resistance

Fungi are a diverse group of eukaryotes, ranging from single-celled yeasts to complex, multicellular moulds (More et al., 2010). Fungal communities are adaptable and may evolve resistance following antifungal exposure (Revie et al., 2018; Ksiezopolska and Gabaldón, 2018; Perlin et al., 2017; Anderson, 2005; Odds et al., 2003). Despite the increasing rates of antifungal drug resistance, just under £1.5 million is allocated to AFR research annually, receiving 'less than 2% of the UKs annual public and philanthropic infection biology research budget' (Barnes et al., 2014). Of particular concern is the absence of therapeutic antifungal alternatives, in comparison to the broad range of antibiotic classes available. This is owing to the position of animals and fungi on the tree of life, revealing close evolutionary relatedness (Hill et al., 2015). Whereas bacteria are distantly related to their animal hosts (Cowen et al., 2001; Doolittle, 1999), animals and fungi are sister clades, leaving few drug targets unique to fungi, and not to their hosts (Fairlamb et al., 2016; Anderson, 2005).

2.3. Antifungal resistance in the environment

Antifungal compounds are widely prescribed in human and/or veterinary medicine and can be present as antimycotics in personal care products, such as antidandruff shampoos (Richter et al., 2013). In addition, a range of fungicides including azole antifungals are commonly adopted as plant protection products ('PPP'), e.g., pesticides, in agricultural applications. There are therefore a number of pathways for antifungals to enter the environment, including the direct usage in agriculture and/or subsequent agricultural run-off (Monapathi et al., 2018). Indirect routes primarily involve wastewater, such as hospital wastewater (Chen and Ying, 2015; Escher et al., 2011; Lindberg et al., 2010) or domestic sewage and biosolid amended soils (Assress et al., 2021, 2020, 2019; Chen et al., 2014; Peng et al., 2012; Kahle et al., 2008).

The AFR selective potential of antifungals at environmentally relevant concentrations is not yet clear. However, there are key findings available to suggest that the environment may drive the evolution of AFR and may also play a role in human exposure to drug resistant opportunistic fungal pathogens. For example, Snelders et al. (2008) reported that 50-71% of Aspergillosis cases in the Netherlands were caused by azole-resistant strains in antifungal naïve patients, suggesting environmental exposure as the source of AFR infection. The European Centre for Disease Prevention and Control's European Environment and Epidemiology ('E3') Network is currently investigating the role of environmental azole use in driving AFR (Perlin et al., 2017).

The 'One Health' approach to tackling AMR is inclusive of human, environment and animal health (Chowdhary and Meis, 2018). Evidently, the environment can play a contributing role in the emergence and spread of resistance across all One Health sectors. Initiatives exist to improve antifungal stewardship in the farming community to try and reduce levels of antifungal pesticides in the environment, such as antifungal agent rotation and the reduced application of higher doses, rather than more frequent and lower doses (Azevedo et al., 2015). Additionally, hrough its National Action Plan, the UK Government has recognised the role of the environment in AMR as an 'area requiring further research' (Courtenay et al., 2019).

2.4. Antifungal monitoring, risk assessment and MSCs

Current antifungal ecotoxicity assessments (e.g., pesticide risk assessments) are based on standardised tests, designed to determine a broad range of endpoints including lethality, growth, reproductive toxicity and endocrine effects on non-target organisms e.g., bacteria, plants, invertebrates and fish (Chen et al., 2014; Richter et al., 2013; Shi et al., 2012; Kjærstad et al., 2010; Haeba et al., 2008; OSPAR Commission, 2005).

The standard approach employed to assess the environmental risk of drugs utilises predicted no effect concentrations ('PNECs') and predicted environmental concentrations ('PECs') (Assress et al., 2020; Roos et al., 2012). European regulations dictate that an

ERA is required where a drug's PEC exceeds 10ng/L (Le Page et al., 2017; EMA, 2006). These predictive values are then used to generate a risk quotient ('RQ'), which may be used to 'quantitatively estimate ecological threat of an aquatic chemical pollutant' (EU Commission, 2003).

It is notable that antifungal resistance is not an endpoint that is currently considered within these assessments. Additionally, it is still unclear whether existing ecotoxicological endpoints are protective of AFR selection at environmentally relevant concentrations (Le Page et al., 2017).

Selective concentration endpoints are determined as the MSC. Gullberg et al. (2011) first conceptualised the experimental determination of the MSC for antibiotics, using a competition-based, single species evolution assay. There have been a number of noteworthy publications since, building on both the definition of MSCs (Table 1) and experimental optimisation for antibiotics.

Table 1. Definitions present in the literature of MSCs for resistance selection.

MSC definition	Use	Reference(s)
'The lowest antibiotic concentration at which the growth rate of resistant and susceptible bacteria are equal.'	Antibiotics	Murray et al. (2020) Gullberg et al. (2011)
'The lowest concentration of antibiotic where the resistant mutant is enriched over the susceptible strain.'	Antibiotics	Stanton et al. (2020)
'The lowest concentration of an antibiotic at which resistance is positively selected, which can be significantly lower than the minimum inhibitory concentration (MIC).'	Antibiotics	Murray et al. (2018)
'The lowest concentration that will select for AMR.'	Antimicrobials	Le Page et al. (2017)
'The minimum concentration at which the presence and expression of resistance gene(s) give bacteria a fitness advantage over non-resistant cells of the same species/strain.'	Antibiotics	Le Page et al. (2017)
'The lowest concentration of an antimicrobial at which positive selection for resistance occurs.'	Antimicrobials	Murray (2017)

‘An estimated selective endpoint, determined by calculating selection coefficients based on change of resistance gene prevalence over time.’	Antibiotics	Murray (2017)
‘The drug concentration where the fitness cost of the resistance plasmid is balanced by the selective effect of the added drug.’	Antibiotics	Gullberg et al. (2014)
‘Where the fitness cost of the resistance is balanced by the antibiotic-conferred selection for the resistant mutant.’	Antibiotics	Gullberg et al. (2011)

In the absence of the empirical determination of antifungal MSCs and lack of definition available for the MSC of antifungal agents, this report recommends and henceforth adopts the definition: the lowest concentration of an antifungal at which positive selection for AFR occurs (adapted from Murray, 2017).

2.4.1. The Watch List

In 2015 (amended in 2018 and 2020) the European Commission generated a Water Framework Directive (‘WFD’) ‘Watch List’ of ‘10 priority substances or groups of substances which are potentially detrimental to the aquatic environment and require better monitoring’ (Loos et al., 2018). The inclusion of antifungals on the Watch List (‘WL’) has helped to raise interest in these agents, including their AFR selective potential. There are currently ten azole antifungals on the WL (Table 2).

Table 2. Azole antifungals listed on the 3rd EU Watch List (Gomez Cortes et al., 2020)

Group	Name	Use
Antimicrobial pharmaceuticals: azole pharmaceuticals (antifungal agents)	Clotrimazole	Human medicine Dermatological – antifungals for dermatological use
	Fluconazole	Human medicine Antimycotics for systemic use
	Miconazole	Human medicine Dermatological- antifungals for dermatological use
PPPs and biocide: azole compounds	Imazalil (enilconazole)	PPP
	Ipconazole	PPP
	Metconazole	PPP
	Penconazole	PPP
	Prochloraz	PPP
	Tebuconazole	PPP and biocide
	Tetraconazole	PPP

2.5. The ‘SELECT’ method

Previous works have developed approaches to empirically determine MSCs for antibiotics (Stanton et al., 2020; Murray et al., 2018; Kraupner et al., 2020, 2018; Lundstrom et al., 2016; Gullberg et al., 2014, 2011). However, many of these methods rely on expensive genotypic analysis or laborious methods.

To address the scarcity of inexpensive and simple experimental options available to generate selective endpoint data for antibiotics, Murray et al. (2020) developed a novel, short-term assay that enables empirical MSC determination for antibiotics. In brief, the SELECT method exposes wastewater derived complex communities of bacteria to a gradient of antibiotic concentrations. This generates selection concentrations that can be used to derive PNECs for resistance (‘$PNEC^{R}$s’), using a significant reduction in growth of a community of bacteria as a proxy for selection for AMR (Murray et al., 2020). The SELECT method has been shown to be robust to changes in community inocula and growth conditions (Murray et al., 2020).

This method is currently optimised to quantify antibiotic effects on complex bacterial communities. However, as previously highlighted, there are currently no methods available to quantify antifungal selective effects on fungal communities. Therefore, this report will explore potential adaptations to the SELECT method, and suggest additional experimental evolution assays with potential to generate antifungal MSCs.

These assays may identify antifungal concentrations at which there is the potential for selection of resistance. Such endpoints may be considered in risk assessments, meaning thresholds can be derived that take into account resistance selection and thus aid the interpretation of antifungal concentrations in the environment.

3. Key considerations for assessing antifungal resistance in the environment

The Codex Alimentarius Commission ('CAC') (2011) describes the key principles 'specific to risk analysis for foodborne antimicrobial resistance' (Ashbolt et al., 2013). Though the CAC is designed with human health risk assessment in mind, it provides a thorough basis to highlight the key information involving AMR selection. Ashbolt and colleagues provide an adapted set of guidelines 'for a human health-oriented risk assessment of environmental antibiotic resistant bacteria ('ARB')':

1. 'Clinical and environmental surveillance programs for antibiotics, ARB and their determinants'
2. 'Epidemiological investigations of outbreaks and sporadic cases associated with ARB, including clinical studies on the occurrence, frequency, and severity of ARB infections'
3. 'Identification of the selection pressures required to select for resistance'
4. 'Human, laboratory, and/or field animal/crop trials addressing the link between antibiotic use and resistance'
5. 'Investigation of the characteristics of the ARB and their determinants'
6. 'Studies on the link between resistance, virulence, and/or ecological fitness (e.g., survivability or adaptability) of ARB'
7. 'Studies on the environmental fate of antibiotic residues in water and soil and their bioavailability associated with the selection of ARB'
8. 'Existing risk assessments of ARB and related pathogens'

Thus, the following section of this report aims to follow these eight adapted CAC principles as broad guidelines to summarise what is currently known and important to consider for an inclusive assessment of antifungal agents, with a particular focus on the risk of AFR selection. Where necessary, comparisons to antibiotics or bacteria will be made.

3.1. Types and uses of antifungals

There are currently only three primary classes of antifungal agents used in the clinic: echinocandins, azoles and polyenes (Ksiezopolska and Gabaldón, 2018). Of these, the azoles are the only class used in both human/veterinary medicine and in agriculture (Fisher et al., 2018). Resistance to just one antifungal drug class reduces therapeutic options by at least 33% and, in extreme cases, resistance to all classes has been observed (Berman and Krysan, 2020). Prophylactic use of antifungal drugs is also widely employed (Assress et al., 2021), resulting in further emergence of resistance in the clinical setting (Revie et al., 2018).

3.1.1. Mode of action ('MoA')

Between the primary antifungal classes, MoAs are somewhat similar (Table 3). Drugs consistently impair cell structure and rigidity by means of interaction with either cell wall or cell membrane constituents.

Table 3. Major antifungal drug classes, MoA and targets (adapted from Berman and Krysan, 2020; Ksiezopolska and Gabaldón, 2018; Anderson, 2005).

Compound class	Drug example	Mode of Action	Target
Azoles	fluconazole	Inhibition of ergosterol biosynthesis	Lanosterol 14α-demethylase
Echinocandins	caspofungin	Inhibition of β-1-3-glucan synthesis	Cell wall, β-1-3-glucan synthase
Polyenes	amphotericin B	Binds to ergosterol	Ergosterol

3.1.2. Azoles

The azoles are the largest class of antifungal agents and can be split into two subsets: imidazoles and triazoles (Odds et al., 2003). Examples of azole pharmaceuticals include fluconazole and miconazole, and azole fungicides include tebuconazole and metconazole (Table 2).

Despite variable chemical structures of azole antifungals, all compounds in this class interact with and target the ergosterol biosynthesis pathway (Moye-Rowley, 2020). Specifically, all azoles inhibit lanosterol demethylase ('LD') (Table 3). This enzyme is responsible for the synthesis of ergosterol, a key sterol constituent of fungal cell membranes (Chen and Ying, 2015; Odds et al., 2003). Therefore, azole exposure results in ergosterol depletion (Hof, 2008). A reduction in ergosterol disrupts the fungal cytoplasmatic membrane structure, modifying membrane-bound enzymes, cell fluidity and decreases growth (Azevedo et al., 2015; Odds et al., 2003; Cowen et al., 2002; White et al., 1998). LD enzymes are encoded by the *erg11* gene in yeasts and the *cyp51* gene in moulds (Perlin et al., 2017; Sagatova et al., 2015; Chowdhary et al., 2014; Morschhäuser et al., 2007).

3.1.3. Echinocandins

In brief, β-1-3-glucan is an essential fungal cell wall component (Berman and Krysan, 2020). β-1-3-glucan is synthesised by the enzyme β-1-3-glucan synthase, which is encoded by the genes: *fks1* and *fks2* (Chaabane et al., 2019). The MoA of echinocandin

compounds is to inhibit this enzyme, which results in a depletion of glucans in the cell wall (Chaabane et al., 2019).

3.1.4. Polyenes

Polyenes, the oldest of the antifungals, act by binding to ergosterol. Unlike the inhibition of ergosterol synthesis caused by azoles, this leads to the creation of 'concentration-dependent channels that kill cells by allowing ions and other cellular components to escape' (Perlin et al., 2017).

3.2. Occurrence, frequency and severity of AFR infections

Diverse fungal communities are present both in the clinic and the environment (Limon et al., 2017). Of concern, numerous fungal species are responsible for invasive infections driving high mortality rates, with *Candida* yeasts and *Aspergillus* moulds the leading human fungal pathogens (Gow and Yadav, 2017; Hill et al., 2015). Collectively, fungal infections cause more annual deaths than malaria, tuberculosis, breast or prostate cancer (Van Dijck et al., 2018; Barnes et al., 2014).

3.2.1. *Candida*

Candida yeasts are known to cause clinically important mucosal diseases, such as thrush, oesophagitis and invasive candidiasis (Berman and Krysan, 2020; Holloman, 2017). In extreme cases, infections may be fatal (Holloman, 2017). Though present as harmless commensals in healthy humans, *Candida albicans* (*C. albicans*) infections carry particularly high mortality rates (nearly 40%) (Gow and Yadav, 2017; Hill et al., 2015).

3.2.2. *Aspergillus*

These predominantly soil-borne moulds are saprophytic and play an essential role in both the carbon and nitrogen cycle (Gisi, 2014). However, the *Aspergillus* genera are responsible for over 90% of human fatalities associated with fungal infections (Brown et al. 2012). *Aspergillus fumigatus* (*A. fumigatus*) is ubiquitous in nature. This species disperses millions of airborne conidia during fungal growth, which are present in most human environments in concentrations up to 200 conidia/m^3 (Gisi, 2014; Meneau and Sanglard, 2005). Once inhaled, conidia may penetrate into the alveoli, resulting in the development of aspergilloma as aspergilli grow within the lung cavity (Verweij et al., 2009). Without being effectively removed, the infection may progress into Invasive Aspergillosis ('IA'). IA is notoriously hard to treat and is often fatal, with mortality rates up to 75% (Cao et al., 2021).

3.3. Selection pressure

So far, there have been no empirical attempts at determining selective concentrations of antifungal agents. Recent works have achieved this for antibiotics and their effects on bacteria and this report aims to draw upon these bacterial examples to assess if the same can be achieved for antifungals.

3.3.1. Evidence from antibiotics

The 'traditional selective window' hypothesis dictates that selection for resistance will only take place at antimicrobial concentrations above the minimum inhibitory concentration (MIC) of susceptible strains (MIC_{SUSC}), and below the MIC of resistant strains (Murray et al., 2018, Gullberg et al., 2011). However, many data for antibiotics challenge this assumption, revealing selection at very low antibiotic concentrations in both single species and complex microbial communities (e.g., Murray et al., 2020, 2019, 2018; Stanton et al., 2020; Kraupner et al., 2018; Le Page et al., 2017; Gullberg et al., 2014; Gullberg et al., 2011; Liu et al., 2011). Sub-inhibitory antibiotic concentrations were first shown to drive selection for resistance by Gullberg et al. (2011), using an increase in the numbers of resistant bacteria as a proxy for positive selection. This study revealed that antibiotic concentrations below the MIC_{SUSC} by up to 230-fold can select for *de novo* mutations and enrich pre-existing resistance (Gullberg et al., 2011).

3.3.2. Evidence from antifungals

Though there are no experimental MSC data currently available for antifungals, Bengtsson-Palme and Larsson (2016) generated a large data set of predicted no effect concentrations for resistance ('$PNEC^R$s') by applying an assessment factor ('AF') to publicly available European Committee on Antimicrobial Susceptibility Testing ('EUCAST') MIC data (Bengtsson-Palme and Larsson, 2016). This data was dominated by clinically important antibiotics, though, seven antifungal pharmaceuticals were also included (Table 4).

To exemplify how such data may be applied to inform safe release limits, the estimated $PNEC^R$s were compared to chemical detection data from WWTP effluent. This revealed that 28% of effluent concentrations exceeded the $PNEC^R$, highlighting the importance of such selective endpoints in detecting selection risk of environmentally relevant concentrations of micropollutants (Bengtsson-Palme and Larsson, 2016).

Bengtsson-Palme and Larsson (2016) note that, whilst this extensive data may aid in informing regulatory practice, the $PNEC^R$s should eventually be supplemented by empirically determined MSCs (Le Page et al., 2017; Murray, 2017).

Table 4. Selection PNEC^{R}s already determined for antifungal agents. Data extracted from *C. albicans* MIC (EUCAST) derived PNEC^{R}s (PNEC$^{R}_{MIC}$) (Bengtsson-Palme and Larsson, 2016).

Antifungal	MIC (*Candida albicans*, EUCAST)	PNEC$^{R}_{MIC}$ (µg/L)
Amphotericin B	1	0.016
Anidulafungin	0.03	0.016
Fluconazole (†)	4	0.24
Itraconazole	0.06	0.008
Micafungin	0.016	*
Posaconazole	0.06	*
Voriconazole	0.25	*

*: indicates zero, †: listed on 3rd EU Watch List.

Assress and colleagues (2021) performed an elaborate monitoring assessment on eight azole antifungals in surface waters and wastewater in South Africa. Based on RQ calculation, this work found that the concentrations of antifungals present posed no high risk against aquatic organisms (algae, daphnia and fish), using traditional ecotoxicity endpoints. However, using the PNEC^{R}s generated by Bengsston-Palme and Larsson (2016), concentrations of fluconazole and itraconazole were found 'to pose moderate to high risk for development of antifungal drug resistance' (Assress et al., 2021; Bengsston-Palme and Larsson, 2016). This confirms concerns that ecotoxicologically derived endpoints may not be protective of resistance selection.

3.4. Linking antifungal use and resistance

Antibiotic resistance selection in bacteria may take place in the environment due to genetic mutations or uptake of antibiotic resistance genes ('ARGs'), under selective and co-selective pressures (Ashbolt et al., 2013; Qiu et al., 2012; Taylor et al., 2011). Likewise, it is widely accepted that persistent antifungal pressure may lead to selection of resistance, with an increased likelihood of selection observed if the agent exerts a fungistatic effect (Azevedo et al., 2015; Hof, 2008). The continual release of clinical azoles from wastewater treatment plants ('WWTPs'), coupled with their frequent and widespread use in agriculture suggests the role of the environment in the emergence of azole resistance is currently underestimated. For example, depending on the compound, three azole fungicide applications are typically made each year to field crops e.g., cereals and potatoes, but

applications may increase after periods of heavy rainfall (Gisi, 2014). This ongoing antifungal exposure creates opportunities for resistance acquisition.

Similar to antibiotic resistance, AFR may be either primary (intrinsic) or secondary (acquired) (Perlin et al., 2017; Ben-Ami et al., 2017). Primary resistance includes strains that are inherently resistant to antifungal drugs. For instance, fluconazole is not active against moulds (Azevedo et al., 2015). Acquired resistance typically follows exposure to antifungal selective pressures, causing mutations, genome rearrangements and resistance determinant overexpression (summarised in Table 5) (Berman and Krysan, 2020; Chaabane et al., 2019; Lockhart et al., 2017).

3.4.1. Case study 1: drug-resistant river borne yeast pathogens in South African communities (Monopathi et al., 2017)

Recent work isolated diverse yeast communities, including *Saccharomyces cerevisiae* (*S. cerevisiae*), from rivers of human and agricultural importance in South Africa. Resistance to priority azole pharmaceuticals, such as fluconazole, was also found. Opportunistic yeast pathogens can cause life threatening mucosal infections in immunocompromised patients, such as HIV positive patients (Yamaguchi et al., 2007). It is estimated that over 10% of the total population of South Africa are HIV positive (Statistic SA, 2014). To reduce anticipated infections, fluconazole is increasingly prescribed prophylactically (Truter and Graz, 2015; dos Santos Abrantes et al., 2014; Morschhäuser, 2002). A large proportion of ingested azole drugs, including fluconazole, are not fully metabolised in the human body, resulting in large quantities entering WWTPs, where compound degradation is equally unsuccessful (Kümmerer, 2008).

The poor performance of WWTPs is especially prevalent in the South African province: North West Province (‘NWP’), meaning concerning quantities of antifungals are emitted into the environment. Prolonged exposure to fluconazole at sub-inhibitory levels may drive environmentally acquired resistance in aquatic yeasts. Consequently, direct exposure of immunocompromised HIV patients to these strains is likely to occur on a daily basis, with a heavy societal reliance on such aquatic systems for recreation, religious and health (food and water) activities. The myriad of increased therapeutic and agricultural use, with insufficient wastewater treatment is likely resulting in high concentrations of both antifungal residues and azole-resistant yeasts in NWP aquatic ecosystems. Here, increased exposure risk and infection may ensue in an already vulnerable immunocompromised population. Such interactions and outcomes will continually increase in combination with one another and is cause for great concern.

3.4.2. Case study 2: *A. fumigatus*

Agriculturally important plant fungal pathogens and *A. fumigatus* share the same environment (Verweij et al., 2009). Consequently, *A. fumigatus* populations are increasingly exposed to azole fungicides, driving an emergence of resistant *A. fumigatus*

('RAF') (Lockhart et al., 2020; Chen et al., 2020; Riat et al., 2018; Berger et al., 2017; Abdolrasouli et al., 2015; Chowdhary et al., 2014; Snelders et al., 2011). Further evidence generated by an extensive monitoring survey in Eastern China identified that RAF prevalence was 'positively correlated ($p < 0.0001$) with residual levels of azole fungicides in soils' (Cao et al., 2021). Bromley et al. (2014) provided additional evidence of the environmental origin of RAF, by identifying greater ($p < 0.05$) RAF in field isolates in comparison to urban isolates.

The consistent finding of a single azole resistance mechanism in RAF, the 34-bp tandem repeat ('TR'), provides strong evidence to link environmental antifungal exposure to the evolution of resistance (Verweij et al., 2009, Snelders et al., 2009). The insertion of this TR is an elaborate process, most likely to occur during sexual reproduction (Jeanvoine et al., 2020; Snelders et al., 2009). Sexual reproduction in *A. fumigatus* almost exclusively occurs in the environment (Jeanvoine et al., 2020).

3.5. Antifungal resistance determinants

As reported above (3.1.1.), each antifungal drug class operates under different MoAs. Similarly, fungal resistance mechanisms and determinants vary according to drug class and, sometimes, species. Examples are given in Table 5. It is key to note that this is not an exhaustive list of either mechanisms or determinants, as there may be further means of resistance to be discovered. Furthermore, given that azoles are the sole class deployed both in human/veterinary medicine and in agricultural practices, more detail is provided for azole specific mechanisms (Verweij et al., 2009).

Table 5. Antifungal drug classes, mechanisms of resistance and their determinants. Mechanisms listed here are relevant to *Candida,* unless indicated otherwise.

Drug class	Mechanism of resistance	Reference(s)
Azoles	Increased efflux pump activity.	Berman and Krysan (2020) Wasi et al. (2019) Perlin et al. (2017) Rocha et al. (2017 Holmes et al. (2016) Shao et al. (2016) Sanglard (2016) Azevedo et al. (2015) Coleman & Mylonakis (2009) Gygax et al. (2008) Hof (2008) Gbelska et al. (2006) Odds et al. (2003)
	erg11 gene product overexpression.	Chaabane et al. (2019) Morschhäuser et al. (2016) Azevedo et al. (2015) Sanglard (2016) Li et al. (2015) Flowers et al. (2012)

		Dunkel et al. (2008)
	erg11 mutations.	Chowdhary et al. (2018) Healey et al. (2018) Azevedo et al. (2015) Hof (2008)
	cyp51 mutations.	Jeanvoine et al. (2020) Chen et al. (2020) Ballard et al. (2019) Riat et al. (2018) Azevedo et al. (2015) Bader et al. (2015) Vincent et al. (2013)
	Biofilm formation.	Berman and Krysan (2020) da Silva et al. (2020) Sav et al. (2018)
	Amino acid substitution (*S. cerevisiae*). Amino acid change (*A. fumigatus*).	Perlin et al. (2017) Ren et al. (2017) Hollomon (2017) Chowdhary et al. (2013) Snelders et al. (2009) Anderson (2005)
	cyp51A gene product overexpression (*A. fumigatus*).	Jeanvoine et al. (2020) Ren et al. (2017) Hollomon (2017) Azevedo et al. (2015) Chowdhary et al. (2013) Snelders et al. (2009)
	Whole-chromosome aneuploidy.	Berman and Krysan (2020)
	Metabolism modifications.	Chaabane et al. (2019)
	TRs (*A. fumigatus*).	Assress et al. (2021) Cao et al. (2021)
Echinocandins	Mutations in hotspot regions of fks*; fks1* for all *Candida* spp, *fks2* in *N. glabrata*.	Fraser et al. (2020) Jiménez-Ortigosa et al. (2017) Perlin et al. (2017) Borghi et al. (2014) Hof (2008)
	Biofilm formation.	Ceballos Garzon et al. (2020) Berman and Krysan (2020) Pristov & Ghannoum (2019) Perlin et al. (2017)
Polyenes	Decreased membrane ergosterol.	McCarthy et al. (2017) Hof (2008)
	erg mutations.	Alpizar Sosa (2020) Carolus et al. (2020) Hof (2008) Cernicka and Subik (2006)

3.5.1. Efflux pumps

The predominant mechanism of azole resistance in *Candida* is the induction of multidrug efflux pumps (Berman and Krysan, 2020; Perlin et al., 2017). Efflux pumps are transmembrane proteins able to actively transport drugs outside of the cell, thus reducing intracellular drug concentrations (Chaabane et al., 2019; Perlin et al., 2017; Jeanvoine et al., 2020). ‘ATP Binding Cassettes’ (ABC) and ‘Major Facilitator Superfamily’ (MFS) are

the two main efflux pump families (Chaabane et al., 2019; Perlin et al., 2017). This resistance mechanism confers resistance to most azoles of both agricultural and medical importance (Gisi, 2014; Hof, 2008).

3.5.2. *erg11* upregulation and mutation in *Candida*

The MoA of all azole drugs targets the ergosterol biosynthesis pathway (Cowen et al., 2002). As previously noted, ergosterol is a key membrane component in fungi (Chaabane et al., 2019). The LD enzyme mediates the synthesis of ergosterol and is encoded by the gene *erg11* (Chaabane et al., 2019; Sanglard, 2016; Cowen, 2001). LD enzymes are found across all biological kingdoms and are biochemically assigned to the enzyme group 'cytochrome P450' (Scott et al., 2020; Caramalho et al., 2017; Hof, 2008). LD enzymes are considered the most ancient cytochrome P450 (Scott et al., 2020). Azole resistance has previously been identified due to an upregulation of the *erg11* gene, resulting in a overexpression of LD despite drug treatment (Chaabane et al., 2019). In addition, point mutations of *erg11* have also been reported to confer azole resistance (Perlin et al., 2017).

3.5.3. Mutation of *cyp51A* in *A. fumigatus*

Changes to the amino acid sequence of the *cyp51A* gene is the most commonly reported mechanism of azole resistance in *A. fumigatus* (Perlin et al., 2017; Ren et al., 2017), present in over 90% of clinical RAF isolates (Verweij et al., 2009). It is speculated that this mechanism is due to the extensive use of azole fungicides in agriculture and has been identified in both clinical and environmental isolates from 22 countries (Perlin et al., 2017). As above, this gene encodes the LD enzyme, but is specific to *A. fumigatus* (Assress et al., 2020; Prigitano et al. 2019). Though there are many mutations documented to be involved, Cao et al. (2021) found that three single nucleotide polymorphisms ('SNPs') (namely TR46/Y121F/T289A, TR34/L98H, and TR53) accounted for 84.8% of all azole resistance mechanisms in *A. fumigatus*.

3.6. Antifungal resistance and fitness costs

The longstanding assumption with AMR evolution is that the selective pressure i.e., antibiotic/antifungal concentration must be large enough to offset any incurred fitness cost of resistance (Melnyk et al., 2015). However, this is based on the false idea that resistance always has a fitness cost (Gullberg et al., 2011). In general, it is suggested that selective effect concentrations are conditional upon the fitness cost of resistance (Gullberg, 2014), and the maintenance of resistance in a population is dependent upon fitness cost in the absence of the drug (Hill et al., 2015; Gullberg, 2014; MacLean et al., 2010; Gagneux et al., 2006; Anderson, 2005).

3.6.1. Bacteria

Generally, antibiotic resistance (particularly acquired) is associated with a fitness cost (Andersson and Hughes, 2010). For example, the alteration of an antibiotic target may prohibit its original function (Enne et al., 2005). Fitness costs may be negated by subsequent evolution or compensatory mutations (Durão et al., 2015; Andersson and Hughes, 2012; Enne et al., 2005; Baquero, 2001). On the other hand, there is evidence to suggest that there are even resistance genes that confer a fitness advantage (e.g., Michon et al., 2011).

3.6.2. Fungi

Comparatively less is known about the fitness cost of AFR in fungi than about antibiotic resistance costs in bacteria. Similar to bacteria, there is evidence that further evolution may take place to ameliorate any incurred fitness costs with compensatory mutations (Verweij et al., 2016; Morschhäuser, 2016; Sharma et al., 2015; Anderson, 2005; Barker et al., 2004). For example, experimental populations of *C. albicans* have previously revealed an initial fitness cost associated with upregulated gene expression owing to the high energy demands of this process, but this cost was reduced over time (Hof, 2008; Cowen et al., 2002). In other research, however, it appears drug resistance sustains no immediate fitness cost (Valsecchi et al., 2015; Chowdhary et al., 2013; Mavridou et al., 2013; Anderson, 2005) or confers great cost with no evidence of compensatory mutations (Vincent et al., 2013). In rare cases, overexpression of resistance determinants, such as ABC transporters, have led to a gain in fitness with and without drug presence (Guo et al., 2017). Specifically, many drug transporters are not specific to azole fungicides and so intracellular concentrations of other toxic compounds may also be reduced (Hof, 2008).

Notably, the sparsely available evidence highlights two potential areas where fitness costs of AFR may be experienced: reproductive output and virulence.

3.6.2.a. Reproductive output

By exposing three different experimental populations of the yeast *S. cerevisiae* to increasing concentrations of fluconazole (16, 32, 64 and 128 µg/ml), Anderson and colleagues (2003) aimed to assess the mean fitness cost of resistance evolution. Fitness was quantified ‘by measuring change in the proportion of strains in mixed [susceptible and resistant] cultures over time’ (Anderson et al., 2003). After propagation of 100 generations via serial transfer, an increase in number of doublings of resistant strains (used to quantify reproductive output as a proxy for fitness) was observed at both 32 and 64 µg/ml. Though, at the highest experimental drug concentration (128 µg/ml), a reduced fitness was witnessed in association with mutations in the gene *pdr1.* This gene encodes for an efflux pump of the ABC transporter superfamily (Anderson et al., 2003).

Calculation of fitness cost in *C. albicans*, conveyed as an increase in doubling time in the absence of fluconazole, has also been documented (Cowen at al., 2000). In this work, the

doubling time was ‘expressed as the number of doublings of the evolved competitor minus the number of doublings of the genetically marked ancestor, standardized by the total number of doublings in the competition assay’ (Cowen et al., 2002). This was calculated during the exponential growth phase using a spectrophotometer (Cowen et al., 2000). All experimental populations were shown to adapt to the presence of fluconazole, measured by an increase in MIC. In addition, this work revealed the initially identical populations followed different doubling times when the presence of fluconazole was removed, and any fitness costs were compensated with further evolution (Cowen et al., 2000). To briefly summarise the findings, a MIC of 4 µg/ml significantly increased fitness, both with and without drug. Where the MIC exceeded 64 µg/ml, there was a considerable increase in fitness with the drug, but no change without. However, where the MIC was 64 µg/ml, a significant reduction in fitness without the drug was found.

3.6.2.b. Virulence

It is postulated that acquired AFR may result in reduced virulence, although, this is still under investigation. To test this, *Candida glabrata* (recently renamed *Nakaseomyces glabrata*; *N. glabrata)* isolates were cultured from a fatal case of recurrent fungemia, which had been unsuccessfully treated with echinocandin therapeutics (Borghi et al., 2014). The studied strains were isolated sequentially from the patient and each strain showed varying echinocandin susceptibility patterns. To assess whether the resistant strains (indicated by an increase in MIC) expressed an associated fitness cost, the invertebrate host model *Galleria mellonella* (*G. mellonella*) was exposed to strains *in vivo* and a correlation between MIC and virulence (conferred by fatal outcome) was calculated. The study concluded that the increase in resistance was not associated with a decreased virulence, shown by a similar rate of killing across the three isolates ($p > 0.05$) (Borghi et al., 2014).

On the other hand, AFR acquisition has been shown to have a virulence cost. For example, experimental populations of *C. albicans* conferring *fks1* mutations revealed reduced virulence (Ben-Ami et al., 2011). Additionally, resistant *C. albicans* mutants have previously been identified to develop fitness trade-offs that influence macrophage susceptibility, host survivability and morphogenesis – key virulence traits (Hill et al., 2015).

At the opposing extreme, resistance mechanisms have been found to be advantageous in terms of virulence. For instance, *in vitro C. albicans* azole-resistant mutants were found to produce greater quantities of extracellular aspartic proteinases, key virulence factors (Rapala-Kozik et al., 2018; Fekete-Forgács et al., 2000). Subsequently, these hyper-virulent resistant strains were found to be more pathogenic in mice hosts than the azole-susceptible parent strain (Fekete-Forgács et al., 2000).

3.7. Environmental occurrence, treatment and persistence of antifungals

As previously stated, azoles are the only antifungal class deployed in both human/veterinary medicine and in agriculture (Chowdhary and Meis, 2018; Berger et al., 2017; Meneau and Sanglard, 2005). Most agricultural azoles are approximately 10-100 times less intrinsically active than their therapeutic counterparts (Gisi, 2014).

However, fungicides are directly applied to agricultural crops at effect concentrations (Gisi, 2014). According to manufacturer guidelines, azoles should be applied at around 100g/ha, which is the estimated equivalent of 10mg of azole fungicide applied per $1m^2$ of plant surface (Azevedo et al., 2015). The spraying of fungicides typically occurs at least once every year (Lago et al., 2014), with nearly 50% of the total European Union (EU) cereal and vineyard acreage treated with azole fungicides (Verweij et al., 2009). In the UK, over 250,000 kg of azole fungicides are utilised for crop protection each year (Chowdhary and Meis, 2018; Kleinkauf et al., 2013).

Fungicide PPPs are essential in the protection of vital crops e.g., wheat, barley and maize, against diseases responsible for reducing crop yields up to 20% (Vanni et al., 2004). With diseases predicted to increase due to climate change (Zubrod et al., 2019), and no effective alternatives to azole fungicides currently available, it is critical to monitor and limit these impacts to secure future food security.

3.7.1. Occurrence

Due to advancements in sensitive and reliable methods for quantifying antifungal concentrations e.g., solid phase extraction, measured environmental antifungal concentrations are available (Appendix A) (Huang et al., 2010). Antifungal residues have been identified in a variety of aquatic ecosystems, including wastewater influents and effluents and surface water, mainly in European or Asian countries (Appendix A). In a comprehensive review conducted by Chen and Ying (2015), environmental levels of azoles were found to be relatively comparable between different countries. In general, concentrations tend to be reduced in surface water due to dissipation, and concentrations appear to be greater in dry seasons than wet due to the dilution effect (Chen and Ying 2015; Chen et al., 2014, Huang et al., 2013). In addition, antifungal concentrations are typically lower in WWTP effluent than influent (Chen and Ying, 2015).

3.7.2. Wastewater treatment

Much like antibiotics, antifungal drugs (e.g., fluconazole) are not fully metabolised in the human body and are therefore excreted and enter the wastewater treatment system (Assress et al., 2021, 2020, 2019; Monapathi et al., 2018; Chen and Ying, 2015; Peng et al., 2012; Kahle et al., 2008). In addition, antimycotics, such as climbazole, are used in cosmetic and personal care products (Richter et al., 2013). Many of these agents are

applied to the human body topically, resulting in greater emissions of active ingredients (90-95%) into WWTPs (Assress et al., 2021, 2020, 2019; Richter et al., 2013; Peng et al., 2012; Letzel et al., 2009). WWTPs do not remove such compounds completely and azole concentrations up to 0.5 μg/L have been identified in WWTP effluent (Richter et al., 2013). For instance, Kahle et al. (2008) quantified the occurrence of nine antifungals in WWTPs and surface waters in Switzerland. This revealed incomplete removal of fluconazole, propiconazole and tebuconazole, but a high removal (80%) of clotrimazole. In particular, fluconazole remains largely unaffected by WWTPs 'due to its hydrophilic properties and low biodegradation rate', resulting in a high prevalence of fluconazole in aquatic systems (Peng et al., 2012; Lindberg et al., 2010; Kahle et al., 2008).

3.7.3. Persistence

In addition to discharge to the aquatic environment from WWTPs, antifungal contamination of sewage sludge is common (Chen et al., 2013b). Owing to the extensive half-life durations and high hydrophobicity of many of these compounds, they may remain and accumulate in the environment for long periods of time i.e., months to years (Table 6). It has previously been hypothesised that sludge application to agricultural land is largely responsible for soil contamination of azoles, in addition to the direct application of azole fungicides (Chen et al., 2013a). Azole fungicides are lipophilic, increasing the presence of residual contaminants in biosolids/sewage sludge (Lai et al., 2014; Wu et al., 2010; Kahle et al., 2008; Boxall et al., 2006). Sludge application to agricultural land as fertiliser is considered a sustainable practice across the world, including in the UK (Chen and Ying, 2015; Langdon et al., 2012). Further to this, reclaimed wastewater is increasingly recycled for irrigation purposes in some countries, which could potentially further increase the contamination of azoles into agricultural land, although this is so far unexplored (Calderon-Preciado et al., 2011, Chen et al., 2011).

Table 6. Soil half-life data for antifungals included on the 3rd EU Watch List.

Compound	Half-life in soil (days)	Reference
Clotrimazole	29-126	García-Valcárcel and Tadeo (2012)
	365	Chen et al. (2013b)
Fluconazole	73-85	García-Valcárcel and Tadeo (2012)
Miconazole	130–440	Chen et al. (2013a)
	347	Walters et al. (2010)
	1386	Gottschall et al. (2012)
Tebuconazole	200	Bhagat et al. (2020)
		Kahle et al. (2008)

3.8. Ecotoxicity testing

There are no universal ecotoxicity tests employed that consider risk for AMR selection. Therefore, the following section aims to summarise the existing ecotoxicity tests for both antibiotics and antifungals, to provide a basis of what is currently available using alternative endpoints.

3.8.1. Antibiotics

There are currently three main ecotoxicity tests employed for antibiotics: the soil nitrogen transformation test, the activated sludge respiration inhibition test ('ASRIT'; OECD, 2005) and growth inhibition of cyanobacteria test (Le Page et al., 2017; Murray, 2017; Brandt et al., 2015). Regardless of the omission of AMR selection from current ERA guidelines, there is further concern owing to the biased nature of the tests towards metazoan species i.e., invertebrates and fish, rather than target microbial species (Le Page et al., 2017; Ågerstrand et al., 2015, Brandt et al., 2015). Le Page and colleagues (2017) questioned the applicability and value of the ASRIT test in quantifying effects of antibiotics on environmental bacteria and called for a reconsideration of its use in ERA.

3.8.2. Antifungals

Ecotoxicity testing of antifungals for the purposes of, for example pesticide and biocide approvals, includes the consideration of traditional endpoints, including developmental toxicity, reproductive toxicity, neurotoxicity, immunotoxicity and oxidative stress (Bhagat et al., 2021). These tests have been previously used to generate PNECs based on traditional endpoints and risk quotients based on available monitoring data for Watch List antifungals (Table 7). Fungal specific toxicity tests are not generally undertaken and not in relation to assessment of the selection for resistance, and it is proposed that these traditional PNEC data may not be protective of AFR selection (Le Page et al., 2017).

Table 7. Available PEC and PNEC data for 3rd EU Watch List antifungals and fungicides (Gomez Cortes et al., 2020)

Compound	PECfw† (µg/l)	MEC (P95*) (µg/l)	PNECfw†	RQfw† (MEC (P95) or PEC/PNEC)
Clotrimazole‡	0.086 (OSPAR, 2015)	0.016	0.02	0.8
Fluconazole‡		0.06	0.613 (Zhou et al., 2019)	0.1
Imazalil (enilconazole)	0.001-0.43 (EFSA; 2010)	0.01 – 0.075	0.8 (Carvalho et al., 2016)	0.09
Ipconazole	0.1088-0.2719 (EFSA, 2013)		0.044 (AgriTox ANSES, 2019)	2.5-6.1
Metconazole	0.1–1.2 (EFSA, 2006)	0.025	0.0291	0.86
Miconazole‡	0.032 (Minguez et al., 2016)		0.4 (Minguez et al., 2016)	0.079
Penconazole	0.184-3.3 (EFSA, 2008)	0.05	6 (INERIS, 2012)	0.008
Prochloraz	0.1-3 (EFSA, 2011)	0.05	0.161 (Zhou et al., 2019)	0.3
Tebuconazole	0.543 – 1.131 (EFSA, 2014)	0.05	0.24 (Swiss ECOTOX Centre, 2016)	0.21
Tetraconazole	2 - 3 (EFSA, 2008)	0.05	1.9 (Lettieri et al., 2016)	0.03

*: 95th percentile, †: freshwater organisms, ‡: clinical antifungals.

4. Modifications to the SELECT method

Without assays to empirically determine antifungal selective concentrations, ERAs for antifungals are unable to consider the risk of AFR selection. One option is to consider whether the SELECT method (Murray et al., 2020), which was developed to determine resistance selection concentrations for antibiotics, could be adapted to consider antifungals.

To consider the adaptation of the SELECT method, this section aims to answer the following questions:

- 4.1. The SELECT method exposes a sewage derived community of bacteria to incremental concentrations of antibiotics. Are there fungal communities present in sewage that will enable SELECT for AFR to be used with this inoculum?

- 4.2. Though complex communities are more representative of the natural environment, working with single species may initially be easier to test the applicability of SELECT to fungi. What are the suggested focal species?

- 4.3. What are the benefits of using single-species vs communities in experimental assays?

- 4.4. The experimental design of the SELECT assay is similar to protocols described in MIC generating broth microdilution assays. Have broth microdilution assays been previously used for antifungal susceptibility testing ('AFST')?

- 4.5. The SELECT method 'determines effect concentrations based on the reduction of bacterial community (wastewater) growth' (Murray et al., 2020). Can a reduction of growth be used as a proxy for AMR in fungi?

4.1. Fungal sewage communities

The use of a sewage derived inoculum in the original SELECT is advantageous as the sewage microbiome is known to be 'representative of both the human gut, hospital effluent and WWTP influent' (Murray, 2017). Importantly, this provides a diverse community of bacteria and resistance mechanisms for selection to act upon, including *de novo* mutation but also horizontal gene transfer ('HGT') of resistance genes between bacteria. For fungi, this may be less important as HGT does not occur and most AFR will be conferred by mutation or increased gene expression.

However, evidence suggests both the diversity and importance of fungal sewage assemblages (Niu et al., 2017). For instance, fungi are the predominant decomposers in WWTPs and form critical components of activated sludge, aiding WWTP management (Gómez-Silván et al., 2020; Zhang et al., 2018). In addition, wastewater associated fungi are not as closely or regularly monitored as bacterial faecal indicator organisms ('FIOs')

and therefore the abundance of such species is likely underestimated (Assress et al., 2019).

Data suggests that yeasts are the most dominant fungi isolated from wastewater (Van Wyck et al., 2012). For instance, Kacprzak et al. (2003) found that yeasts made up 97.5% of sampled wastewater fungal communities, predominantly represented by *Candida* spp. Interestingly, wastewater contamination by yeasts, such as *Candida*, has been compared to that of bacterial FIOs, such as faecal coliforms (Monapathi et al., 2020; Brizzotti-Mazuchi et al., 2020; Monapathi et al., 2017; Hagler et al., 2017). Some authors have suggested yeasts could be adopted in association with faecal indicator ('FI') bacteria as bioindicators of aquatic pollution (Brizzotti-Mazuchi et al., 2020). Previously, correlations have been demonstrated between levels of FI bacteria and yeasts (Van Wyck et al., 2012; Medeiros et al., 2008). For example, in a study assessing the prevalence of fungi in parallel with bacterial FIOs in Greece, counts of yeasts were significantly ($p < 0.01$) correlated with those of total faecal coliforms (Arvanitidou et al., 2002).

Due to the presence of antifungals in wastewater systems, WWTPs have also been identified as sources of AFR fungi (Assress et al., 2021; Niestępski et al. 2019; Ohore et al. 2019). Previously, to isolate fungal communities from wastewater samples, 1 ml of water samples were spread onto selective agar and plates were incubated at 28°C for 7–10 days. Colonies were then picked and further inoculated in nutrient rich media (Assress et al., 2021). Various additional methods to isolate yeast from wastewater have previously been described, yet the most utilised protocol involves filtering wastewater through a cellulose membrane, followed by subsequent culturing on agar plates (Brizzotti-Mazuchi et al., 2020).

Importantly, sewage and aquatic systems are not the only environment of importance in terms of potential for AFR evolution. Fungal soil communities are well recognised for both ecological importance and as hotspots of AFR e.g., RAF. Hence, assays to determine antifungal MSCs in fungal soil communities are explored in section 5.

The available evidence therefore indicates that diverse fungal communities are present in WWTPs. In addition, there are protocols to enable selective culture of yeast species from wastewater. These findings indicate that wastewater 'is a good medium for the growth and sporulation of different groups of fungi' (Kacprzak et al., 2003). Following culturing protocols outlined in the literature (e.g., Assress et al., 2021), yeasts are easily isolated from natural samples on selective agar, identified by morphology and this allows single isolates to be used as inocula for further experimentation. Furthermore, owing to the ubiquity and ecological flexibility of *Candida* yeasts in such environments (Silva-Bedoya et al., 2014), this report will focus on adapting the SELECT method for *Candida* spp. Notably, Murray and colleagues (2020) validated the MSCs determined by the SELECT method against changes to inoculum and experimental conditions e.g., temperature. Therefore, it is recommended that antifungal MSCs derived by the antifungal adapted SELECT should also be validated against changes to inocula and temperature.

4.2. Suggested fungal species

When considering a model species for the purpose of this report, there are a few key considerations to address. As highlighted, *Candida* yeasts are ubiquitous in sewage-derived communities, so initial modifications could consider this focal genus. In addition, AFR and invasive infections of therapeutic importance are also well documented in *Candida* spp. (Bhattacharya et al., 2020; Chaabane et al., 2019; Whaley et al., 2017). Therefore, the species within this genus are of epidemiological importance.

Table 8. Key differences between bacteria, yeast and mould.

Bacteria	Yeast	Mould
Unicellular	Unicellular	Multicellular
Asexual reproduction	Asexual reproduction	Asexual & sexual reproduction
Non-hyphal	Non-hyphal	Hyphae (filamentous)
Non-sporous & sporous	Non-sporous	Sporous
Short cultivation time	Short-medium cultivation time	Medium-long cultivation time

Importantly, there are key differences in the growth, structure and function of yeast cells in comparison to moulds (summarised in Table 8). These differences impact the organism's ability to evolve and maintain AFR, and therefore impact our ability to quantify resistance selection. Notably, similarities between yeast and bacteria may be drawn upon, including that they are both unicellular, undergo asexual reproduction and have typically short cultivation times. Therefore, as the SELECT method is currently optimised to test antibiotic selective effects on a sewage derived bacterial community, application to yeast cells is more likely to yield similar quantification abilities for resistance acquisition. Furthermore, as will be later discussed, yeast cells are routinely utilised in broth microdilution AFST assays. These assays follow similar protocols to the SELECT method. Though broth microdilution is possible for moulds, inocula typically consist only of hyphal fragments or conidial suspensions, suggesting optical density ('OD') may not be adequate to measure growth of moulds in response to antifungals.

4.2.1. Evolution of resistance in fungi versus bacteria

In addition to the marked differences between eubacterial genetics and growth, versus eukaryotic physiology, there are clear differences in the evolution of resistance between fungi and bacteria (summarised in Table 9). One of the most obvious differences is the absence of fungal capacity to readily take up or horizontally transfer exogenous DNA, such as plasmids (Azevedo et al., 2015; Hof, 2008). This suggests resistance development in fungal communities may be more gradual, as opposed to the 'explosive expansion of resistance' observed in bacteria (Hof, 2008). Furthermore, some resistant bacterial strains are able to produce enzymes capable of degrading antibiotics. This mechanism of resistance is most notably documented with the extended spectrum beta-lactamase ('ESBL') producing bacteria, such as ESBL-producing *E. coli* (Salinas et al.,

2021). On the contrary, fungal enzymes able to degrade azoles have not yet been identified (Hof, 2008).

Nevertheless, fungi have been described as 'evolvable' (Cowen et al., 2002), owing to the large number of genes encoding for resistance mechanisms e.g., efflux pumps (Berman and Krysan, 2020). For example, there are 30 known genes for ABC transporters present in *S. cerevisiae,* providing greater possibility for resistance mutations to arise (Hof, 2001; Cowen, 2001). Moreover, fungal genomes are significantly larger than bacterial genomes (Table 9). This has been proposed to increase the likelihood of genetic mutations conferring antifungal drug resistance.

Table 9. Summary table comparing evolution of resistance in bacteria and fungi.

Resistance mechanism	Bacteria	Fungi	Reference(s)
Target modification e.g., mutation	Yes	Yes	Chaabane et al. (2019) Cortés et al. (2019) Chowdhary et al. (2018) Luthra et al. (2018) Healey et al. (2018) Azevedo et al. (2015) Hof (2008)
Inhibition of target access (pores, pumps) e.g., efflux	Yes	Yes	Berman & Krysan (2020) Wasi et al. (2019) Yılmaz et al. (2017) Perlin et al. (2017) Rocha et al. (2017 Holmes et al. (2016) Shao et al. (2016) Sanglard (2016) Azevedo et al. (2015) Coleman & Mylonakis (2009) Gygax et al. (2008) Hof (2008) Gbelska et al. (2006) Odds et al. (2003)
Enzymatic modification/inactivation of antimicrobial compound	Yes	No	Gasparrini et al. (2020) Schaenzer & Wright (2020) Markley & Wencewicz (2018) McCarthy et al. (2018) Hof (2008)
Capacity for evolution of resistance	**Bacteria**	**Fungi**	**Reference(s)**
Horizontal gene transfer	Yes	No	McInnes et al. (2020) Sun et al. (2019) Fairlamb et al. (2016)

			Azevedo et al. (2015)
Size of genome	Smaller e.g., *E. coli* 4640kb	Larger e.g., *S. cerevisiae* 12,068kb	Hokken et al. (2019) Cowen et al. (2002)
Rearranging of genomes	No	Yes	Stukenbrock & Croll (2014) Cowen et al. (2002)

One way to reduce dissimilarities between fungal and bacterial resistance evolution is to consider ploidy. Ploidy is a fundamental genetic feature of all organisms, traditionally defined by the number of chromosome homologues present in a cell (Gerstein and Sharp, 2021; Trun, 1998). According to this definition, the majority of bacteria, including universally adopted FIO *E. coli*, 'contain one homologue of their single chromosome' and may therefore be considered haploid (Bull, 2019; Trun, 1998).

Fungal ploidy is not as simple (Gerstein and Sharp, 2021). For example, *C. albicans* is the most widely investigated fungal pathogen for resistance to antifungals (Lee et al., 2020). However, unlike *E. coli*, *C. albicans* is diploid, meaning they contain two homologues or copies of each chromosome (Glazier and Krysan, 2020). With a diploid background, the degree of dominance of a mutation is important to be considered in experimental evolution assays (Cowen et al., 2002). For instance, point mutations in one recessive chromosome may be compensated by the unaffected, dominant allele (Hof, 2008), thereby preventing/reducing the appearance of the expected phenotype.

Genetic exchange and recombination within haploid genomes are much more clear-cut (Cowen et al., 2002) and would provide a closer model system to that of predominantly coliform bacteria employed in the existing SELECT method. Two haploid yeast species of epidemiological and environmental importance are *N. glabrata* and *Candida auris* (*C. auris*) (Shor and Perlin, 2021; Du et al., 2020; Kumar et al., 2019). Therefore, these two species are recommended, discussed further below, to trial the application of the SELECT method to measure the selective effect of azole antifungals.

4.2.2. *N. glabrata*

N. glabrata are amongst the most common yeasts isolated from water sources (Monapathi et al., 2020, 2017; Van Wyck et al., 2012). These yeasts can also exhibit multidrug resistance ('MDR') to azoles and echinocandins (Farmakiotis and Kontoyiannis, 2017; Healey et al., 2016; Pfaller, 2012; Singh-Babak et al., 2012). This species is well documented for increasing spread of resistance and is therefore of clinical concern (Azevedo et al., 2015; Srinivasan et al., 2014). Many (~50%) *N. glabrata* isolates have a hypermutator phenotype because of defects in DNA mismatch-repair machinery, making this species particularly 'evolvable' (Perlin et al., 2017; Farmakiotis and Kontoyiannis, 2017). However, it has been noted that *N. glabrata* can 'behave unreliably and cannot be relied upon for standardised testing' (Perlin et al., 2017). For example, Espinel-Ingroff et

al. (2013) compared caspofungin MICs for *N. glabrata* and observed high interlaboratory variability.

4.2.3. *C. auris*

C. auris is a recently discovered *Candida* species, first described in 2009 (Arikan-Akdagli et al., 2018). The global spread of this fungal pathogen is concerning for several reasons (Perlin et al., 2017). *C. auris* has been highlighted as the first 'globally emerging fungal pathogen that exhibits MDR' with a 'strong potential for nosocomial transmission' (Farmakiotis and Kontoyiannis, 2017). *C. auris* strains are also prone to cause outbreaks owing to high inherent resistance to antifungal agents (Chaabane et al., 2019; Farmakiotis and Kontoyiannis, 2017). For example, out of 54 *C. auris* clinical isolates sampled across three continents, over 90% were resistant to fluconazole and over 40% exhibited MDR (Lockhart et al., 2017; Farmakiotis and Kontoyiannis, 2017). In addition, *C. auris* was recently isolated from coastal environments in India, including salt marsh and sandy beach sites, supporting an ecological relevance of this species (Arora et al., 2021).

4.2.4. Fungal species conclusion

It is likely that *N. glabrata* is the most widespread and may therefore provide a more environmentally representative species. However, as *C. auris* has only recently been described, evidence of environmental abundance may not be truly accurate. In addition, a variability of data from MIC testing in of *N. glabrata* has been highlighted, arguably owing to the species' hypermutability (Espinel-Ingroff et al., 2013; Perlin et al., 2017). For these reasons, it is suggested that wild type isolate strains of *C. auris* may be the most suitable focal species to initially test the SELECT method with antifungals.

Importantly, both *N. glabrata* and *C. auris* have been documented to exhibit intrinsic resistance to drugs of clinical importance. Intrinsic resistance is frequently observed across both yeast and mould species, adding an additional challenge when prescribing effective medication to fungal infections. Therefore, experimental inocula should be determined on a case-by-case basis, based on the available information, to ensure antifungals are not tested against a species that is intrinsically resistant to that compound.

It should also be noted that haploid genomes are not representative across all fungal species, meaning MSC variability may be observed for species with diploid backgrounds e.g., *C. albicans.* Hence, once the antifungal SELECT method has been established for the proposed haploid species, it is recommended that further testing or adaptations should be considered to enable application to diploid species, such as *C. albicans.*

4.3. Single species versus community

Both single species and community assays have different strengths for various experimental applications (summarised in Table 10).

Table 10. Strengths proposed in the literature for single species and community-based assays for both bacterial and fungal species/communities.

Single species	Complex community
Minimise genetic drift (Cowen et al., 2000)	'Microorganisms are not commonly encountered in isolation' (Assress et al., 2019)
'Valuable insights regarding the potential of antibiotics to select for resistant strains in environments characterised by low bacterial complexity and favourable growth conditions' (Kraupner et al., 2018)	Most bacteria and fungi exist predominantly in organised communities in nature (Van Dijck et al., 2018)
Ability to replicate experiments (Cowen et al., 2002)	'Ability to predict what occurs in natural environments' (Murray, 2017)
Control conditions such as ploidy (Cowen et al., 2002; Mable and Otto, 2001)	'Complex interactions occur in a natural community' (Murray, 2017; Berglund et al., 2015).
Control size of population (Cowen et al., 2002; Wahl and Krakauer, 2000; Zeyl, 2000)	'MSCs derived in single species are unrepresentative of MSCs determined in a community' (Klumper et al., 2019; Murray, 2017; Brandt et al., 2015; Berglund, 2015)
	Considers factors such as 'the combined effects of changes in community structure, protective morphological forms and alternative selection pressures e.g., nutrient limitation and predation' (Le Page et al., 2017; Lundström et al., 2016; Bengtsson-Palme and Larsson, 2016; Balcázar et al., 2015; Day et al., 2015; Gullberg et al., 2014).
	Provides insights into the development of AMR in environmentally realistic scenarios (Le Page et al., 2017)

It is evident from the literature that the adoption of a community-based approach provides environmental realism and gives greater predictive power. However, single species assays are less complex, facilitating thorough characterisation of the test system and generating data with lower variance.

In the interest of initial modifications of the SELECT method, experimental trials should first utilise single species populations of either *C. auris* or *N. glabrata* for the reasons already discussed. However, to improve realism of single species assays, strains of the same species with variable resistance patterns can be included. For example, Kraupner et al. (2020) recently exposed an artificial mix of *E. coli* strains with variable resistance patterns to hospital and municipal wastewaters to assess selection potential for resistant bacteria. This accounts for competition between different strains and is likely more representative of 'real world scenarios' (Kraupner et al., 2020). Therefore, the use of mixed fungal isolates with varied resistance profiles is recommended.

These mixed communities could be generated in the laboratory. Or, future research could prioritise trialling complex sewage-derived yeast communities, with the use of a natural sewage community as a potential source of diverse fungal isolates. Additional important environmental compartments of fungal communities e.g., soil may also provide potential sources of fungal communities, especially predominantly soil-borne *A. fumigatus.* Assays to determine selective effects of antifungals on soil associated *A. fumigatus* are discussed in section 5.

4.4. Broth microdilution and yeasts

Broth microdilution assays are widely used to determine MICs for antimicrobial agents. The MIC 'is the lowest concentration of an antimicrobial agent that prevents or inhibits the visible growth of fungal cells, as established by a standardised endpoint' (Van Dijck et al., 2018). The general principle employed in these assays is to measure the growth of a known inoculum of microorganisms suspended in a nutrient medium, in the presence of incremental concentrations of antimicrobials (Van Dijck et al., 2018).

The universal protocols adopted for such assays are provided by the Clinical and Laboratory Standard Institute ('CLSI') in the United States and EUCAST in Europe (Borman et al., 2017; Arendrup et al., 2017; Pfaller et al., 2014; Wayne, 2008). Methods are proposed for both yeasts and filamentous fungi, such as *A. fumigatus.* These protocols generate data with high agreement and differences between the described methods are minimal (Berman and Krysan, 2020; Pfaller et al., 2014). However, one difference of importance to the purpose of this report concerns the determination of MIC values. For the CLSI method, MIC values are determined by visual inspection ('the eyeball method'), which can be inaccurate and leaves opportunity for subjectivity between laboratories (Dalarze and Sanglard, 2015). On the other hand, EUCAST protocols determine MIC values more accurately using spectrophotometric endpoint optical OD measurements (530nm), which are advantageous in providing a quantitative measure of growth inhibition (Van Dijck et al., 2018; Delarze and Sanglard, 2015).

In summary, broth microdilution assays are widely used to determine antifungal effects on yeasts. These growth-based assays illustrate cellular responses to antifungal agents by quantifying growth to determine susceptibility (Srinivasan et al., 2014). Given that these

protocols are established and validated to determine susceptibility profiles of yeast, it could be suggested that this supports the adaptation of the SELECT method to determine selective effect concentrations of antifungals on yeasts.

Using these protocols, conceptual alterations of the original SELECT parameters are provided, to accommodate optimum conditions for yeast cultures (Table 11).

Table 11. Recommendations of SELECT modifications

Criteria	SELECT (Murray et al., 2020)	Suggested alterations derived from broth microdilution (EUCAST)
Readings	Every hour for 12 h up to 60 h	Every hour for 12 h up to 60 h
Broth	Iso-Sensitest™ broth‡	RPMI 1640 medium‡
Temperature	37°C	35°C
Community	Washed sewage	Single species mixed susceptibility culture e.g., resistant and susceptible *C. auris* strains
Shaken at	120 rpm	Unknown§
Starting drug conc.	EUCAST MIC* CBP†	EUCAST MIC* CBP†, or where necessary, predetermine MIC for agricultural azoles
OD	600nm	530 nm

*: MIC – minimum inhibitory concentration. †: CBP – clinical breakpoint. ‡: Iso-Sensitest was chosen for use in the SELECT method due to its low binding affinity to antibiotics. RPMI 1640 media is beneficial for fungal susceptibility testing and generates *in vitro* data with a strong correlation to *in vivo* scenarios. Further systematic studies are required to inform the optimum media selection for environmental monitoring. §: Further testing is required to inform optimum conditions for environmental monitoring.

4.5. Growth as a proxy for AMR in fungi

The core principle behind the SELECT method is that it determines selective effect concentrations of antibiotics 'based on a reduction of bacterial community growth' (Murray et al., 2020). Therefore, the following section aims to summarise key findings in the literature to support the hypothesis that growth may be used as a proxy for AMR selection in fungi.

4.5.1. Optical density measurements

Like broth microdilution AFST assays, the SELECT method exposes a community of bacteria to concentration ranges of antibiotics in a 96-well plate, using OD measures to determine no observed effect concentrations ('NOECs').

A similar method was employed in a study where susceptibility profiles across a range of *C. albicans* growth rates were compared between planktonic and sessile/biofilm cells (Bailie and Douglas, 1998). Significantly, this study revealed that planktonic *C. albicans* were only resistant to polyene drug, amphotericin B, at low growth rates (≤0.13 h^{-1}) and drug susceptibility was overall highly dependent on growth rate (Bailie and Douglas, 1998). To measure growth rate, OD measurements (540nm) were taken in a continuous culture outflow. These findings suggest that a reduction in growth may indeed be a proxy for AFR selection.

Similarly, though optimised for fungal biofilms, a novel growth assay used OD readings (600nm) to determine the number of *C. albicans* cells present in biofilms (Lohse et al., 2017). This method performed 384-well standard OD assays in the presence of caspofungin or amphotericin B (Lohse et al., 2017). The authors note that OD readings correspond to viable cell counts and can therefore be used to measure growth in response to antifungals. Additionally, benefits of this approach include being less labour intensive, providing consistent results and allowing high throughput application (Lohse et al., 2017).

This suggests that a reduction in growth could be used as a proxy for selection for AFR in yeast. However, it is key to highlight that the evidence to support this is sparsely available through lack of research. Therefore, it is crucial to consider accompanying genotypic validation assays to empirically determine if observed growth reduction in a SELECT-type assay is associated with the identification of known resistance determinants. Suggestions of validation assays are provided in section 5.

4.6. Conclusions

Here, the findings presented in response to the originally proposed questions are summarised:

- 4.1. Are there fungal communities present in sewage?

- Fungal communities present in sewage are diverse and may harbour AFR strains. A focus on yeast species is recommended, due to their ubiquity and experimental flexibility.

- 4.2. What are the suggested focal species?

- The use of fungal species with haploid genomes for a SELECT type approach, as an initial stage, is recommended. In particular, this report recommends *C. auris,* but where *C. auris* may be intrinsically resistant to test antifungal agents, *N. glabrata* may also be used.

- 4.3. What are the benefits of using single-species vs complex communities in experimental assays?

- In brief, community approaches are more representative of the natural environment. However, for initial SELECT adaptations for antifungals, a single species derived population should be used. Cultured wild-type colonies, both resistant and susceptible can provide initial experimental inocula.

- 4.4. Have broth microdilution assays been previously used for AFST?

- Broth microdilution assays are used widely for AFST and established protocols are described. Suggestions are listed for antifungal SELECT modification (Table 11).

- 4.5. Can a reduction of growth be used as a proxy for AMR in fungi?

- The evidence available in the literature suggests that a reduction of growth may be used as a proxy for AMR in some model yeast species, but validation is required.

5. Validation and alternative methods

The MSCs determined by the SELECT method were initially validated against a previously published, longer-term genotypic assay (Murray et al., 2020, 2018), using quantitative polymerase chain reaction ('qPCR') to quantify prevalence of key resistance gene targets. In brief, sewage bacterial communities were serially cultured for a total of 7 days in the presence of different concentrations of a test antibiotic. Effect concentrations were determined where target gene prevalence was significantly greater than the no-antibiotic control (Murray et al., 2018). Like the SELECT method, this genotypic validation assay is optimised for bacteria. Therefore, this report proposes the following validation assays for an antifungal SELECT-type assay.

In order to measure antifungal selective effects on fungi, validation methods should aim to quantify key resistance mechanisms within populations. The two primary resistance mechanisms observed in AFR include the inhibition of target access e.g., overexpression of efflux pumps (5.1 and 5.2) and target modification e.g., mutations (5.1 and 5.3).

Therefore, the following section will outline assays to determine and measure mechanisms of resistance, including:

- Phenotypic assays (Section 5.1)
- Overexpression (Section 5.2)
- Target site mutations (Section 5.3)

For each sub-section, suggestions of validation assays will be given and supported with examples where these tools have been experimentally applied. Validation methods are suggested for yeast species, specifically *Candida*, given that this is our proposed genus for an antifungal SELECT assay. In addition, these proposals are made with azole antifungals in mind.

However, the emergence of AFR moulds of clinical and environmental importance is a significant global issue. Therefore, it is important to also consider means of determining antifungal effect concentrations for notable mould species. For this reason, alternative methods are also suggested that could be applied to assess antifungal effects on important mould species, focussing on *A. fumigatus.*

5.1. Phenotypic assays

Phenotypic assays, including the SELECT method, have the advantage of not requiring knowledge of pre-determined resistance determinants. Observed resistant strains in phenotypic-based assays will encompass all responsible resistance determinants in the test culture and therefore provide a population wide representation of resistance selection.

5.1.1. Yeasts

In a simple test tube system, Kraupner et al. (2018) established selective concentrations for ciprofloxacin resistance in a sewage effluent bacterial community. Though used for bacterial communities, this method could be modified and utilised to establish selective concentrations for antifungals in single yeast species or even mixed yeast communities.

In Kraupner et al. (2018), *E. coli* inoculated test tubes were supplemented with incremental concentrations of ciprofloxacin and low nutrient media. After 24 hours, cultures were diluted and re-inoculated with fresh broth and antibiotic at the same concentration. At 0-, 24- and 48-hours aliquots of each culture were plated on chromogenic agar, supplemented with or without ciprofloxacin. These were incubated and colony forming units ('cfu') were enumerated. From the cfu data, proportions of phenotypically resistant *E. coli* and total coliforms were calculated. Selective concentrations were statistically determined where ratios of resistant to overall cfu counts were significantly different from the control (Kraupner et al., 2018).

Therefore, this report suggests a similar protocol may provide a low cost, replicable and interlaboratory validation assay for the MSCs determined by the antifungal-SELECT method. There are clear modifications to be made to the method described by Kraupner et al. (2018), such as adoption of a yeast selective agar (e.g., bismuth sulfite glucose glycine yeast agar) and yeast specific nutrient broth (e.g., RPMI 1640 medium) (Assress et al., 2021). A further necessary modification may include alterations of duration of both broth cultivation and agar plate incubation. For example, Assress et al. (2021) suggest a plate incubation time of 7-10 days. This would significantly increase the duration of the assay.

There may also be opportunity to detect cross resistance to multiple antifungal agents via this method. For example, four well plates containing breakpoint concentrations of itraconazole, posaconazole and voriconazole with an additional drug-free control have recently been developed and validated for *A. fumigatus* susceptibility testing (VIPcheck, Nijmegen, Netherlands) (Arendrup et al., 2017). Resistance to azole compounds is conferred where growth is detected in drug-containing wells. This would increase the number of compounds that could be tested and would likely reveal more environmentally relevant data, as environmental yeast communities will be exposed to a diverse mixture of compounds in wastewater and aquatic systems. As noted, this method is currently only validated for *A. fumigatus* and therefore may provide an additional potential approach for deriving selective antifungal concentrations for moulds, such as *Aspergillus,* in addition to

those suggested in the following section. However, this method could also be applied to yeasts, provided appropriate antifungal breakpoints are adopted.

To conclude, by adopting this phenotypic assay over a genotypic alternative, the costs of increased experimental duration may be offset by the significantly reduced consumable costs. Furthermore, there is the advantage of rapidly and simply testing for phenotypic cross resistance to multiple azole agents. However, it should be noted that such phenotypic assays are generally considered less sensitive than the molecular methods outlined in sections 5.2 and 5.3, and therefore could overestimate the MSC.

5.1.2. *A. fumigatus*

A. fumigatus are predominantly soil-borne and therefore require alternative isolation protocols. For example, in order to test for resistance, *A. fumigatus* was recently selectively cultured from soil samples. Briefly, soil samples were suspended in saline solution (0.85% NaCl), then spread on selective agar plates supplemented with the antibiotic chloramphenicol. After incubation, colonies can be morphologically identified as *A. fumigatus* and isolated for further testing (Cao et al., 2021).

Ren and colleagues (2017) performed an elaborate evolution experiment exposing *A. fumigatus* environmental isolates to triazole fungicides. Soil samples were suspended in saline, spread onto Sabouraud's dextrose agar ('SDA') and incubated. *A. fumigatus* isolates were identified by micro- and macroscopic morphologies. Following standard AFST, a random sample of susceptible isolates were further cultured on SDA plates for 7 days, conidia were harvested and suspended in a saline solution. Aliquots of this conidial suspension were added to Sabouraud's dextrose broth medium ('SDBM') containing various fungicides at different concentration and was used to serially culture experimental populations of *A. fumigatus* (Ren et al., 2017). Using a pre-described broth dilution AFST method for filamentous fungi (John, 2008), initial MIC values of unevolved strains were compared to final MIC values of evolved strains. This showed an increase in MICs for a number of strains, for all test antifungals (voriconazole, itraconazole and posaconazole).

This approach could be used to determine selective effect concentrations of antifungals if a range of concentrations were used to evolve isolates, and increases in MICs were determined using standard AFST. In addition, given that soil samples are suggested as the source of fungal species, it could be suggested that an approach such as this may be considered to determine MSC data in fungal soil communities in future work.

5.2. Overexpression

One of the key advantages of using a phenotypic assay is that these assays include all responsible resistance determinants in a community. However, there are well characterised resistance determinants that genotypic assays may target to generate selective endpoint data. However, these methods are often more costly and laborious than the SELECT method.

An important AFR mechanism involves the upregulation of genes encoding resistance mechanisms e.g., efflux transporters, resulting in overexpression of the proteins encoded by those genes. Using a batch microcosm experimental approach similar to that used to validate the SELECT assay (e.g., Murray et al., 2018), effect concentrations of antifungals where expression levels were significantly different to basal control levels could be determined following serial culture in the presence of different antifungal concentrations.

Methods to profile gene expression (Table 12), including reverse transcriptase quantitative PCR ('RT-qPCR'), are extremely sensitive, accurate and are able to rapidly analyse small samples, allowing high throughput generation of results. RT-qPCR quantifies gene expression using messenger ribonucleic acid ('mRNA') (Adams, 2020). However, these methods are significantly more expensive than phenotypic assays e.g., the SELECT method, and variable results between laboratories may be generated due to the multiple RT-qPCR enzymes and oligonucleotides commercially available (Mohamed, 2019; Valasek and Repa, 2005). In addition, the reliability of RT-qPCR based measurements are dependent on normalisation, typically using an internal control housekeeping or reference gene (reviewed in Paul et al., 2020).

5.2.1. Yeasts

Upregulation of genes, including *erg11* and genes encoding for ABC or CDR transporters, is the predominant mode of azole resistance in yeasts (Berman and Krysan, 2020; Perlin et al., 2017). When such genes are upregulated, the proteins encoded by those genes are overexpressed. Experimental approaches to enable quantification of gene expression levels are widely available (summarised in Table 12). Previous research highlights many key gene targets for the major *Candida* species which, when upregulated, confer resistance (Table 13).

5.2.1.a. *erg11*

As noted previously, ergosterol is a key fungal membrane component and is mediated by the LD enzyme, encoded by the *erg11* gene (Chaabane et al., 2019; Sanglard, 2016). Upregulation of this gene has been shown to confer azole resistance (Table 12). *Erg11* is regulated by a transcription factor protein encoded by *upc2* in *Candida* yeasts (Sanglard, 2016; Dunkel et al., 2008). Gain-of-function mutations in *upc2* increase activity and leads to the increased expression of *erg11,* and thus, the LD enzyme will be overexpressed (Morschhäuser, 2016; Flowers et al., 2012).

Using RT-qPCR (Table 13), Flowers et al. (2012) investigated *erg11* expression levels in fluconazole-resistant *C. albicans* isolates. This work identified 75% of test fluconazole-resistant isolates upregulated *erg11* by at least two-fold compared to expression levels in unrelated susceptible strains (Flowers et al., 2012).

5.2.1.b. Overexpression of efflux transport systems

Efflux transport systems actively reduce intracellular drug concentrations and thus, confer drug resistance. Efflux proteins and transporters are often species specific. For instance, at least 18 ABC and 33 MFS transporters are reported for *N. glabrata* (Sanglard, 2016; Gbelska et al., 2006).

Multiple genes encoding transporters in *C. albicans* are well documented, including *cdr1*, *cdr2*, *cdr3*, *cdr4*, *cdr11* and *snq2* (Table 12). In particular *cdr1* and *cdr2* are the most studied (Coleman and Mylonakis, 2009) and their increased expression is documented in several clinical isolates (Sanglard et al., 2009). The regulation of genes encoding ABC and MFS transporters is mediated by specific transcription regulators. For example, *cdr1* and *cdr2* are regulated by a transcription regulator encoded by *tac1.* Mutations in these regulator genes confer a 'hyperactivation state', resulting in an upregulation of genes encoding for efflux transporters, and thus, will results in an overexpression of the associated transporters (Sanglard, 2016; Dunkel et al., 2008).

Importantly, Gygax et al. (2008) developed a RT-qPCR based assay for azole resistance in *N. glabrata*. Expression levels of *cdr1, pdh1* and *pdr1* were consistent among susceptible isolates, with a three-fold change in expression observed in fluconazole resistant isolates. The authors compared this method to the conventional broth microdilution assay for AFST and highlighted the simplicity and efficiency of the RT-qPCR based assay, proposing it as a 'cost-effective method for early detection' (Gygax et al., 2008).

Table 12. Examples of upregulation and overexpression quantification in yeasts

Species	Antifungal	Method	Target gene upregulated	Finding	Reference
C. auris	Fluconazole	RT-qPCR	*cdr1 and cdr2*	Findings support *cdr2* expression maintained by transcription factor encoded by tac*1*.	Li et al. (2021)
	Fluconazole	RT-qPCR	*caur_02725* (orthologous to cdr*1), cdr4*, cdr*6*, and snq*2*	Increased expression of all targets.	Wasi et al. (2019)
	Fluconazole	RT-qPCR	*cdr1*	High-level azole resistance dependent on an ABC transporter encoded by *cdr1.*	Kim et al. (2019)
	Fluconazole	RT-qPCR	*erg11*	*'erg11* expression was inducible *in vitro* with fluconazole exposure'.	Chowdhary et al. (2018)
N. glabrata	Fluconazole	RT-qPCR	*erg9* and *erg11*	Both targets upregulated and important in ergosterol biosynthetic processes.	Alves et al. (2020)
	Fluconazole and amphotericin B	RT-qPCR	*cdr1*	Combination therapy reduced *N. glabrata* growth more than fluconazole monotherapy.	Mohamed (2020)
	Fluconazole	RT-qPCR	*pdr1, erg11, cdr1, cdr2, snq2, yor1, ybt1, qdr2 and rta1*	An approximate three-fold increase in expression of Pdr1 was observed in resistant strains carrying mutations in *pdr1*.	Tantivitayakul et al. (2019)
	Fluconazole, itraconazole and voriconazole	RT-qPCR	*cdr1, cdr2, snq2, erg11, and pdr1*	'Significant upregulation of *cdr1* and *cdr2* (P<0.05), whereas no obvious differences were found for *snq2, erg11*, and *pdr1* (P>0.05).'	Yao et al. (2019)

	Fluconazole	RT-qPCR	*cdr1*	'Resistant strains showed overexpression of Cdr1 compared with sensitive strains.'	Shahrokhi et al. (2017)
	Fluconazole	RT-qPCR	*cdr1, cdr2, snq2* and *erg11*	'*cdr1* overexpression was observed in 57.1 % of resistant isolates. *snq2* was upregulated in 71.4 % of the cases. *erg11* overexpression does not seem to be associated with azole resistance, except for isolates that exhibited azole cross-resistance.'	Gohar et al. (2017)
	Fluconazole and tacrolimus	RT-qPCR	*erg11, cdr1, pdh1* and *snq2*	'The expression levels of the *erg11* and *snq2* genes were significantly downregulated after exposure to the drug combination, whereas that of the *cdr1* gene was significantly upregulated, and no significant change in expression of *pdh1* gene was observed.'	Li et al. (2015)
	Fluconazole	RT-qPCR	*cdr1, pdh1* and *pdr1*	Three-fold change in expression was observed in fluconazole resistant isolates.	Gygax et al. (2008)
C. albicans	Fluconazole and itraconazole	RT-qPCR	*cdr1* and *cdr2*	*tac1* mutations responsible for Cdr overexpression.	Liu et al. (2020)
	Fluconazole	Semi-quantitative RT-PCR	*cdr1, cdr2, mdr1, erg11*	'Many isolates elicited higher expression'.	Kumar et al. (2020)
	Fluconazole	RT-qPCR	*cdr1, cdr2, mdr1, mdr2* and *flu1*	Upregulation of *cdr1, cdr2, mdr1* and *mdr2* was found in resistant isolates. No link between *flu1*	Pourakbari et al. (2017)

				expression and fluconazole resistance.	
	Fluconazole	RT-qPCR	*cdr1, cdr2, mdr1* and *erg11*	*cdr1* overexpression was identified as the major resistance mechanism.	Salari et al. (2016)
	Fluconazole	RT-qPCR	*cdr1, cdr2, mdr1* and *flu1* genes	'Compared with fluconazole-susceptible C. albicans isolates, *cdr1* gene expression displayed 3.16-fold relative increase, which was statistically significant.' No significant difference in other genes.	Zhang et al. (2014)
	Fluconazole	RT-qPCR	*erg11*	Fluconazole-resistant isolates overexpressed *erg11* by at least 2-fold compared to expression levels in unrelated susceptible strains.	Flowers et al. (2012)
	Fluconazole	RT-qPCR	*erg11*	Increase of expression levels in resistant strains, over susceptible counterparts.	Chau et al. (2004)
C. krusei	Itraconazole	RT-qPCR	*erg11, abc2*	'The mRNA levels of *erg11* gene in itraconazole-resistant isolates showed higher expression compared with itraconazole-susceptible dose dependent and itraconazole-susceptible ones.'	He et al. (2015)

5.2.2. *A. fumigatus*

Similar to *erg11* in yeasts, the *cyp51A* gene encodes the central enzymes responsible for ergosterol biosynthesis and is the primary target of azole compounds in *A. fumigatus* (Perlin et al., 2017). The upregulation of *cyp51A* and genes encoding efflux transporters have also been found to cause resistance to azole compounds in moulds (Table 13).

For example, Aruanno et al. (2021) performed a microevolution experiment exposing an azole-susceptible *A. fumigatus* strain to sub-MIC concentrations of voriconazole in successive subcultures. Transcriptomic analyses identified the upregulation of *cyp51A* and several ABC and MFS transporter genes (Aruanno et al., 2021). To calculate expression fold changes, RT-PCR was used.

Following a similar microevolution approach, Cui et al. (2019) induced tebuconazole resistance in *A. fumigatus* isolates serially cultured in a liquid medium. To achieve this, environmental soil isolates of *A. fumigatus* were cultured following protocols outlined by Ren et al. (2017). Subsequently, a conidial suspension was generated and serially incubated in SDBM containing tebuconazole and evolved strains were isolated for analyses. Efflux transporter gene mRNA levels were assessed using RT-qPCR, allowing comparisons of relative expression levels of evolved, wild-type and control strains. Significantly, key efflux pump gene expression levels, including *atrF*, *afumdr1*, *afumdr2*, *cyp51A* and *cyp51B,* of evolved strains were higher compared to the control (Cui et al., 2019).

This sophisticated study provides evidence which suggest that resistance can be experimentally induced *in vitro* in complex mould species, such as *A. fumigatus.* This suggests that an assay similar to that described, monitoring expression levels of yeast cultures at different antifungal concentrations, could be used to derive selective endpoints where mould expression levels are significantly greater than the control.

Table 13. Examples of upregulation and overexpression quantification in *A. fumigatus*

Antifungal/fungicide	Method	Target upregulated gene	Finding	Reference
Voriconazole	RT-qPCR	*erg1*, *erg3*, *erg3A*, *erg24*, *erg24 B*, *erg25, erg25B, abcB, abcD, mdr1, mfsC, mdrA, atrI, afu1g16160*	Increased expression of all targets.	Aruanno et al. (2021)
Itraconazole, posaconazole, voriconazole	RT-qPCR	*mdr1*, *mdr2*, *mdr3*, *atrF*, *cypP51* A and *cyp51*B	Upregulation was mostly observed for resistant isolates.	Mroczynska et al. (2020)
Voriconazole	RT-qPCR	*cyp51A*	Upregulation in resistant strains.	Sturm et al. (2020)
Itraconazole, voriconazole, isavuconazole, Posaconazole	RT-qPCR	*mfsC, cyp51A, cyp51B* and *abcD*	*mfsC* and *abcD* increased expression. No significant upregulation of *cyp51A* or *cyp51B*.	Sharma et al. (2019)
Tebuconazole	RT-qPCR	*atrF*, *afumdr1*, *cyp51A* and *cyp5 1B*	Resistance caused by overexpression.	Cui et al. (2019)
Itraconazole	RT-qPCR	*afumdr3* and *afumdr4*	'Most resistant mutants showed either constitutive high-level expression of both genes or induction of expression upon exposure to itraconazole.'	Nascimento et al. (2003)

5.2.3. Efflux activity

In addition to the quantification of gene expression, there are assays available to evaluate efflux pump activity. The general principle behind such assays involves the addition of fluorescent dyes to a cell suspension, allowing the fluorescence kinetics of efflux to be measured. These have previously been adopted in combination with expression profiling e.g., RT-qPCR assays as a phenotypic validation method, but may be useful for SELECT validation.

5.2.3.a. Yeasts

Bhattacharya et al. (2016) evaluated *C. albicans* efflux pump activity in association with ABC and MFS transporter overexpression using fluorescence assays. To quantify expression levels, RT-qPCR was used. This study adopted two fluorescence assays, namely, alanine-β-naphthylamide ('Ala-Nap') and rhodamine 6G ('R6G') (Bhattacharya et al., 2016).

The Ala-Nap assay provides a more general measure of efflux activity, with application capabilities for both ABC and MFS transporters (Bhattacharya et al., 2016). Ala-Nap is a dye which, when applied according to the outlined Ala-Nap assay protocol (Sherry et al., 2012; Rajendran et al., 2011), indicates increased efflux pump activity by displaying increased fluorescence (Rajendran et al., 2011). The R6G assay enables efflux activity quantification specifically for Cdr1 and Cdr2 transporters (Bhattacharya et al., 2016). Here, efflux values are expressed as fluorescent R6G dye efflux from fungal cells into supernatant (Bhattacharya et al., 2016; Ivnitski-Steele et al., 2009; Nakamura et al., 2001).

Efflux activity assays, such as Ala-Nap and R6G, are advantageous in that they are 'straightforward, quick and cost-effective' (Bhattacharya et al., 2016). Given that high efflux rates and azole drug resistance are shown to be closely correlated, the direct measure of efflux activity provided by these tools may be useful in the rapid identification of selective concentrations of antifungal agents. For instance, following a similar protocol to Murray et al. (2020) for the SELECT method, yeast cell suspensions exposed to different antifungal concentrations in 96-well-plates could also be exposed to the dyes outlined above. Selective concentrations might then be identified where efflux activity, conferred by increased fluorescence, is significantly greater than no antibiotic controls.

This would serve as a valuable validation tool for the SELECT method, but may not be sufficiently accurate as a standalone assay. Importantly, these assays will only detect resistance conferred by increase efflux, whereas the SELECT is inclusive of all resistance determinants.

5.2.3.b. *A. fumigatus*

A. fumigatus transporter activity can also be determined using the same protocol outlined for R6G (Nakamura et al., 2001). For instance, Aruanno et al. (2021) measured drug transporter activity of experimental *A. fumigatus* strains. Additionally, extracellular R6G levels were previously used to measure efflux pump activity of azole-sensitive and -resistant *A. fumigatus* strains (Li et al., 2015). To increase efflux activity, glucose was added to conidial suspensions. It was found that efflux activity was greater in the azole-resistant strain in comparison to the azole-sensitive strain. Therefore, this may provide a simple validation tool to support the quantification of expression levels of efflux transporters.

5.3. Target site mutation

Mutations of target genes can also play an important role in AFR. Advancements in molecular technologies allows the exploration of fungal genomes and enables the identification of important AFR genes and target site mutations (Ball et al., 2020). Whole genome sequencing ('WGS') has been previously employed to identify and investigate mutations that confer AFR *in vitro* (Ball et al., 2020). The adoption of WGS has broadened our understanding and documentation of resistance mechanisms and key resistance mutations responsible. Though more time-consuming and expensive than many of the other methods discussed, sequencing remains the 'gold standard' for mutation detection (Paul et al., 2021; Zhao et al., 2016).

5.3.1. Yeasts

5.3.1.a. Whole genome sequencing

Vincent et al. (2013) used WGS to identify mutations conferring amphotericin B resistance in *C. albicans* isolates. By sequencing drug sensitive parent strains, these can be compared with evolved resistant strains to determine mutations conferring resistance. Hence, a validation assay may involve WGS of control strains in comparison with evolved strains following exposure to a range of concentrations of antifungals.

5.3.1.b. *erg11* mutations

Erg11 encodes the LD enzyme in yeasts, which is responsible for the synthesis of key membrane component, ergosterol (Chaabane et al., 2019). As previously described, the overexpression of this enzyme leads to the increased synthesis of ergosterol and mediates azole resistance. In addition to upregulation of *erg11*, mutations in the coding of this gene are also known to result in amino acid alterations that ultimately confer azole resistance (examples provided in Table 14).

Tetra primer-amplification refractory mutation system-PCR ('T-ARMS-PCR'), restriction site mutation ('RSM'), and high-resolution melt ('HRM') analysis methods are some of the

molecular tools available to determine resistance caused by *erg11* polymorphism. These three assays were recently evaluated by Paul and colleagues (2021) for their ability to rapidly detect resistance determinants in azole resistant *C. tropicalis* isolates, in comparison with susceptible counterparts. Briefly, T-ARMS-PCR is used for SNP genotyping (Etlik et al., 2011). The RSM assay detects mutations 'in the specific target DNA sequence of the restriction enzyme' and has only been adopted once to detect *erg11* mutations (Paul et al., 2021; first described by Steingrimsdottir et al., 1996). Finally, HRM analysis has previously been used to predict azole resistance in *C. albicans* by examining *erg11* polymorphisms (Caban et al., 2016) and enables discrimination of amplified products with 'single nucleotide variation by generating different melting curves after amplification' (Paul et al., 2021).

Paul et al. (2021) identified the T-ARMS-PCR and RSM approaches to be marginally more sensitive in discriminating resistant and susceptible isolates than HRM analysis. The authors conclude by recommending all three methods in their 'specificity, analytical sensitivity, time and cost of analysis' in the 'rapid detection of *erg11* mutations in *C. tropicalis'* (Paul et al., 2012).

Though unable to quantify prevalence of resistance gene polymorphisms, these methods are useful to identify the presence of resistant strains. Therefore, the methods outlined here may serve better as validation tools to the assays outlined, rather than additional means to determine antifungal MSCs.

Table 14. Examples of *Candida* resistance mutations.

Species/**antifungal**	**Method**	**Target gene**	**Finding**	**Reference**
C. tropicalis **Fluconazole**	T-ARMS-PCR, RSM, HRM	*erg11*	'Resistant isolates showed A339T and C461T mutations in the *erg11* gene.'	Paul et al. (2021)
C. glabrata **Fluconazole**	Nucleotide sequences of genes amplified by PCR were compared with sequences deposited in the GenBank database	*erg3*, erg*11* and *pdr1*	'N768D and E818K mutations in *pdr1* co-occur with overexpression of drug transporters.'	Tantivitayakul et al. (2019)
N. glabrata *C. albicans* **anidulafungin, micafungin, caspofungin, fluconazole, posaconazole, voriconazole, itraconazole and isavuconazole**	Next generation sequencing	*erg11, erg3,* *tac1* and *gsc1 (fks1)* in *C. albicans* *erg11, pdr1, fks1* and *fks2* in *N. glabrata*	Identified novel resistance mutations. 'All nine echinocandin-resistant *Candida* isolates showed mutations in the hot spot regions of *fks1*, *fks2* or *gsc1*.' 'Seven-point mutations in *erg11* were determined in azole-resistant *C. albicans* whereas in azole-resistant *C. glabrata*, no *erg11* mutations were detected.'	Spettel et al. (2019)
C. albicans, N. glabrata and *C. parapsilosis* **Azole and echinocandin resistant strains**	Next generation sequencing	*erg11, erg3, tac1, pdr1, fks1* and *fks2*	A total of 391 SNPs were detected, among which 6 SNPs were reported for the first time.	Garnaud et al. (2015)

5.3.2. *A. fumigatus*

Decreased susceptibility to azole treatment has been documented following several known *cyp51A* amino acid changes (Table 15), resulting in a reduced binding affinity of azole compounds (Warrilow et al., 2015). *cyp51A* mutations are reported as the principal source of azole resistance in *A. fumigatus* (Ren et al., 2017). Resistance arising from target site mutations is thought to have been driven by the widespread application of azole fungicides to agricultural land (Bader et al., 2015).

Tandem repeats ('TRs') in the *cyp51A* promotor region are frequently observed in environmental azole resistant strains (Arai et al., 2020). The most common of these include TR sequence of 34 base pairs with L98H mutations (TR_{34}/L98H) and TR sequence of 46 base pairs with Y121F/T289A mutations (TR_{46}/Y121F/T286A) (Arai et al., 2020; Bader et al., 2015). For instance, Bader et al. (2015) PCR amplified and sequenced the *cyp51A* gene of 55 resistant *A. fumigatus* isolates. The majority of resistant isolates (80%) harboured the TR_{34}/L98H allele. TR46/Y121F/T289A variants were the second most frequently observed. Therefore, by PCR amplifying the *cyp51A* gene and sequencing for known mutation variants, resistant and susceptible strains can be differentiated.

Recently, a novel assay was developed to aid the 'rapid detection of *cyp51A* mutations' (Arai et al., 2020). Surveyor nuclease ('SN') assays detect mutations using a mismatching-specific endonuclease SN enzyme (Arai et al., 2020). These assays have previously been adopted to validate the presence or absence of mutations following genome-editing experiments, such as CRISPR/Cas9 (Kang et al., 2015).

The SN assay was verified against 48 azole-resistant and susceptible *A. fumigatus* strains and revealed rapid detection of strains harbouring different *cyp51A* single point mutations (Arai et al., 2020). Arai and colleagues (2020) demonstrated that the assay was able to distinguish wild-type *cyp51A* from point-mutated genes. Authors highlight advantages of this assay, including the rapid determination of the presence or absence of different resistance mutations using only one primer set. However, given that the assay has only recently been described, further validation is required before wider applications are adopted.

Detection of *cyp51A* mutations via conventional PCR amplification or the novel SN assay could be used as a validation tool to determine whether resistant strains are selected for in a population. For example, a resistant phenotype can be validated via the identification of known resistance mutations. Importantly, however, this method can be applied only to detect pre-determined mutations (examples in Table 15).

Table 15. Examples of *cyp51A* mutations conferring AFR in *A. fumigatus*

Antifungal/fungicide	Method	Target gene	Finding	Reference
Itraconazole, voriconazole, tebuconazole and difenoconazole	'Azole resistant strain genomic DNA was extracted. Both the *cyp51A* and promoter region were amplified. The sequence was compared to those of an azole-susceptible strain.'	*cyp51A*	Revealed resistance mechanisms: 'TR46/Y121F/T289A, TR34/L98H, TR34/L98H/S297T/F495I and TR53'.	Cao et al. (2021)
Voriconazole	SN assay	*cyp51A*	'The Surveyor nuclease assay could rapidly detect *cyp51A* mutations with one primer set. Also, all the tested strains harbouring different *cyp51A* single point mutations could be clearly distinguished from the wild type.'	Arai et al. (2020)
Itraconazole	Amplification of entire *cyp51A* gene and its promoter region using PCR to reveal mutations	*cyp51A*	Identified azole-resistant *cyp51A* mutations: M220K, M220I, M220R, G54E, G54W and N248K/V436A, Y433N substitution.	Chen et al. (2019)
Itraconazole, posaconazole and voriconazole	Sequencing of *cyp51A* gene	*cyp51A*	'A G54R mutation was identified in the isolates exhibiting itraconazole and posaconazole resistance, and	Talbot et al. (2018)

			the TR_{34}/L98H mutation in the pan-azole-resistant isolate.'	
Itraconazole, voriconazole and posaconazole	PCR amplification and sequence analysis of *cyp51A* gene.	*cyp51A*	Sequence analysis exhibited mutations: TR46/Y121F/T289A and TR34/L98H/S297T/F495I.	Ren et al. (2017)
Itraconazole	'Conventional PCR assay was carried out to determine the presence of the TR34/L98H mutation in the *cyp51A* gene of triazole-resistant *A. fumigatus* isolates'	*cyp51A*	'Among resistant isolates, TR34/L98H mutations in the *cyp51A* gene were the most prevalent'.	Nabili et al. (2016)
Itraconazole, voriconazole and posaconazole	'A PCR-based assay was developed to screen for the presence of the 34-bp TR in the promoter region of the *cyp51A* gene.'	*cyp51A*	'This study reports for the first time the presence of the TR_{34}/L98H mutation in a UK environmental *A. fumigatus* isolate'.	Bromely et al. (2014)
Itraconazole	'*cyp51A* gene was amplified by PCR and compared to *cyp51A* sequence in GenBank for comparison to detect mutations.'	*cyp51A*	'Showed the presence of two alterations, a 34-bp tandem repeat in the promoter region combined with the presence of a mutation that led to a substitution at codon 98 of leucine to histidine (TR/L98H), 86% of resistant isolates'.	Snelders et al. (2009)

Itraconazole	‘For [resistant] isolates the full coding sequence of both strands of the *cyp51A* gene was determined by PCR amplification and analysed to detect mutations’	*cyp51A*	‘A substitution of leucine 98 for histidine in the *cyp51A* gene, together with two copies of a 34-bp sequence in tandem in the gene promoter (TR/L98H), was found to be the dominant resistance mechanism’.	Snelders et al. (2008)
Itraconazole	‘Molecular analysis included random amplified polymorphic DNA (RAPD) assay and sequencing of the *cyp51A* gene’	*cyp51A*	‘Revealed a mutation of M220I in cytochrome P450 sterol 14-α-demethylase in the second resistant isolate and a mutation of G54R in the last three resistant isolates’.	Chen et al. (2005)

6. Alternative assays requiring further exploration

Modifications of the SELECT method to determine the selective potential of antifungal agents have been suggested. Furthermore, methods that may be applied as SELECT validation tools have been proposed, supported by examples in the literature.

In this section, additional assays that have previously been used to measure growth or virulence are provide. These assays may be modified to provide novel validation methods or alternatives to the SELECT method with further work. The suggestions outlined here are speculative and would require additional testing before application.

6.1. Ergosterol content as a proxy for growth

By upregulating *erg11* and *cyp51A* genes, ergosterol target molecules will increase, meaning a greater dose of antifungal is required, which often results in AFR. In addition, given that the overall MoA of azole compounds inhibits ergosterol biosynthesis, it is possible that ergosterol content of a population of yeast or mould cells may give an indication of growth or survivability in the presence of antifungal agents, and thus predict resistance. Importantly, an assay targeting ergosterol biosynthesis to determine selective endpoints may only be applied to derive azole MSC data, given that echinocandin and polyene MoAs differ in target and mechanisms.

If changes in ergosterol content can be used as a proxy for resistance selection, ergosterol quantification assays could be modified to detect selective endpoints of antifungals. For example, a suspension of yeast or mould cells could be experimentally evolved in the presence of different concentrations of antifungal agents. Selection for resistance may be conferred at the antifungal concentration where ergosterol levels in the culture at the end of the experiment are significantly lower than the no-antifungal control. At this concentration, it is likely that the antifungal causes a fitness disadvantage to susceptible bacteria, therefore indicating a selective pressure leading to resistance emergence - analogous to that conferred by a reduction in growth in the SELECT method.

6.1.1. Yeasts

Arthington-Skaggs and colleagues (2000) first described an assay, entitled the ‘sterol quantitation method’ (‘SQM’), that measures cellular ergosterol content and was proposed to provide AFST alternatives that do not rely on growth inhibition to determine MIC values. Briefly, this method extracts total intracellular sterols from *Candida* by harvesting yeast cells, adds an alcoholic potassium hydroxide solution followed by incubation in a water bath. The sterol extract is then diluted in ethanol and scanned spectrophotometrically, with ergosterol content illustrated by ‘a characteristic four-peaked curve’. This method

generates ergosterol content as a percentage of the wet weight of cells (Arthington-Skaggs et al., 2000).

This method or similar derivations have since been applied in additional experimental systems. For example, Alizadeh et al. (2017) compared ergosterol content and *ERG11* gene expression in competing resistant and susceptible *C. albicans* strains. The resistant and susceptible isolates were exposed to different concentrations of fluconazole for 24 h, then cells were harvested, weighed, lipids extracted, supernatant removed, and OD readings were taken using a spectrophotometer (Santos et al., 2012). Ergosterol content was calculated using the standard ergosterol calibration curve. Data obtained was expressed as 'the percentage of ergosterol in comparison with the untreated controls' (Alizadeh et al., 2017; Santos et al., 2012). Furthermore, Khodavandi and colleagues (2018) adopted this same protocol for spectrometric analysis of ergosterol content in combination with RT-qPCR of *erg11* to analyse the effect of antifungal activity on ergosterol synthesis in MDR *C. albicans* strains.

This simple and rapid quantification of ergosterol content could be adopted as either a validation for the SELECT method, or any of the genotypic assays described here. An assay such as this may also be used as a standalone tool to identify selection for azole resistance, given that all azole compounds interact with ergosterol synthesis.

6.1.2. Moulds

The importance of ergosterol is central to all fungal species, suggesting that ergosterol content could provide a simple method to generate selective endpoints for *A. fumigatus* or other mould species.

Though the original method described by Arthington-Skaggs et al. (2000) was designed for *Candida* yeasts, it has since been applied to *A. fumigatus* cultures. For example, Alcazar-Fuoli et al. (2008) extracted total ergosterol from *A. fumigatus* strains following this protocol, but with minor adjustments. Following growth in a liquid medium, 'mycelia were harvested by filtration', then sterols were extracted following the original assay. Data from this work was expressed as 'micrograms of ergosterol by milligrams of fungal dry weight' (Alcazar-Fuoli et al., 2008).

Hence, this may provide an additional tool to measure the effect of antifungal agents on fungal moulds by quantifying total ergosterol content following antifungal exposure. By exposing *A. fumigatus* conidial suspensions to sub-MIC concentrations of antifungals, ergosterol content of exposed cultures compared with control levels may determine effect concentrations of antifungal agents. As outlined for yeasts, this may provide a standalone tool or a valuable validation assay for other methods proposed.

Evidence recently provided by Ballard et al. (2019) suggested that azole resistance is associated with decreased ergosterol content in *A. fumigatus* fungal membrane. To quantify ergosterol, *A. fumigatus* was grown overnight and collected via vacuum filtration.

Following this, dried mycelia were ground to a fine powder and hydrolysed. Ergosterol was then extracted in hexane and 'ergosterol concentrations were measured by gas chromatography-mass spectrometry' (Ballard et al., 2019). This suggests preliminary tests should be conducted to analyse alterations in ergosterol content in response to different antifungal concentrations, with additional use of genotypic methods to detect where resistance selection occurs.

6.2. Decreased virulence as a proxy for AMR

As highlighted in chapter 3, greater wax moth larvae (*Galleria mellonella*) have previously been used in invertebrate host model assays for fungal infection investigations. This report specifically highlighted the use of *G. mellonella* in work by Borghi et al. (2014), which aimed to explore the associated cost of echinocandin resistance in *N. glabrata* on virulence, expressed as changes in fatal outcome. This work concluded that, owing to a similar rate of killing across resistant isolates with varying susceptibility patterns, increased resistance did not influence virulence (Borgi et al., 2014). However, evidence suggests AFR may impose an associated virulence cost (Hill et al., 2015; Ben-Ami et al., 2011). If reduced virulence may be used as a proxy for AFR in yeast species, the *G. mellonella* host model may be modified to enable detection of antifungal selective endpoints. It is important to note that the relationship between virulence and AFR is not yet understood, therefore this report stresses that this assay suggestion is purely based on speculation.

For example, yeast cell suspensions exposed to different antifungal concentrations could be injected into the *G. mellonella* haemocoel. Using mortality as a measure of virulence and reduced virulence as a proxy for resistance, effect concentrations might be identified where rate of killing is significantly lower than the control.

Mini-host model assays, including *G. mellonella,* have advantages such as its cost and simplicity allowing greater replication of larvae injection (Ames et al., 2017). In addition, this model invertebrate has been utilised for a variety of *Candida* species, including *C. albicans* (Brennan et al., 2002; Cotter et al., 2000), *C. parapsilosis, C. orthopsilosis, C. metapsilosis* (Gago et al., 2014), *C. tropicalis* (Mesa-Arango et al., 2013) and *C. krusei* (Scorzoni et al., 2013).

7. Assay comparison

Both the SELECT method and proposed validation methods have advantages and disadvantages in terms of defining MSC/selective concentrations of antifungals (Table 16).

The modified SELECT method remains the most efficient and cost-effective assay. To verify the SELECT method, validation tools should be selected on a species and agent specific basis. For example, culture-based methods e.g., Kraupner et al. (2018) may be more advantageous for yeast application than for mould. Furthermore, genotypic assays such as targeting *erg11* and *cyp51A* sequencing are only applicable for the relevant fungus e.g., *erg11* for yeasts and *cyp1A* for moulds. Finally, the novel assays proposed e.g., ergosterol content quantification and invertebrate host models, require further testing before application to determine MSCs.

Table 16. Strengths, weaknesses, opportunities and threats analysis of proposed assays.

Approach	Strengths	Weaknesses	Opportunities	Threats
SELECT e.g., Murray et al. (2020)	• Inexpensive, allowing the rapid generation of data • Robust to changes in community inocula and growth conditions • Rivals efficiency and accuracy of traditional methods e.g., ASRIT • Community based assay representative of a complex, mixed sample (sewage) • Highly replicable, but minimal replication (6) shown to be robust • OD provides quantitative measure of growth • Low between replicate variability during exponential growth phase • Captures any competition and selection occurring for all the available resistance genes and mutations present in that community	• Restricted to antibiotics and bacterial inocula • Doesn't consider mixing of antibiotics as occurring in the environment • Does not give information on resistance mechanisms responsible	• Potentially adaptable for other antimicrobial compounds e.g., antifungals, as shown in this report • Can address scarcity of experimental MSC data • Data generated can inform safe release limits and antimicrobial regulatory guidelines • Could enable better investigation into evolution of AMR by defining target selective windows • Could be applied to determine MSCs for polyenes and echinocandins with further investigation and adaptations • Could be used to test mixtures of antimicrobials, but further validation required	• Optimum laboratory conditions e.g., high temperatures and nutrient are not replicable of natural environment and may lead to possible under or overestimation of MSCs – though previous validation has shown minimal effect of growth conditions • May only be applicable to yeast species, not moulds • Concern of high intrinsic resistance in many fungal species so drug-bug combinations must be case specific • The testing of single antimicrobials does not account for co-selection, which has been found to be of importance in environmental settings

	• Viable candidate for validation against OECD test guidelines • Does not limit the MSC estimation to a particular gene or gene class • Applicability as a standalone assay			
Single species batch microcosm - culture based e.g., Kraupner et al (2018), broth microdilution	• Cost effective • Relatively high throughput • Allows rapid and simple replication • Reduced consumable costs • Established protocols shown to have minimal interlaboratory variation in results • Use of commercially available agar plates streamlines process • Interlaboratory replicable • All responsible resistance determinants included	• Limited to culturable microorganisms that can grow in culture media • Does not give information on resistance mechanisms responsible • Use of single species unrepresentative of environment • High nutrient broth and high temperature not environmentally representative • Plating is less sensitive than other methods	• Could allow rapid detection of cross resistance • Use of environmental strains would increase environmental relevance • Can be utilised for both yeasts and moulds, though requires further testing • Nutrient and temperature conditions can be modified to improve environmental realism • Could be applied to determine MSCs for polyenes and echinocandins	• Incubation of fungi significantly longer than bacteria and therefore increases assay duration • Uncertainty in application to mould species and so requires further testing • Negative culture is not sufficient to completely rule out resistance as will only reveal culturable organisms • Concern of high intrinsic resistance in many fungal species so drug-bug combinations must be case specific • As resistant cfu used as endpoint, drug tolerance in fungi could be an issue

				• Optimum laboratory conditions e.g., high temperatures and nutrient are not replicable of natural environment and may lead to possible under or overestimation of MSCs
Overexpression **e.g., RT-qPCR,**	• Extremely sensitive • Rapidly analyse small samples • High throughput generation of results • Accurate measure of specific resistance mechanism • Highlights specific mechanism responsible	• Expensive • Variable results due to different enzymes and oligonucleotides commercially available • Relies on knowledge of resistance determinants • Reliability dependent on normalisation – typically using housekeeping genes – lack of interlaboratory consistency of gene adopted for normalisation • Usually only one resistance target analysed, with each additional target adding further expense • Increase in replicates increases processing	• Can be applied to both yeast and mould species • High nutrient and temperature conditions could be modified to better represent environment	• Optimum laboratory conditions e.g., high temperatures and nutrient are not replicable of natural environment and may lead to possible under or overestimation of MSCs • Doesn't consider mixtures so will not account for co-selection • Will only account for resistance due to overexpression and not target site modifications e.g., mutations • Would require identification of different targets to facilitate application to echinocandins and polyenes

		time – may compromise data quality and cost • High nutrient broth and high temperature not environmentally representative		• Concern of high intrinsic resistance in many fungal species so drug-bug combinations must be case specific
Mutations e.g., WGS, T-ARMs-PCR, RSM, HRM and SN assay	• Conventional methods well established e.g., PCR • Accurate measure of specific resistance mechanism • Highlights specific mechanism responsible • Not limited by pre-existing knowledge of resistance determinants as able to identify novel determinants	• More time consuming and expensive • Many assays proposed are relatively novel and therefore have minimal testing history and validation • Increase in replicates increases processing time - may compromise data quality and cost	• Allows greater exploration of widely undocumented fungal genomes • Can be applied to both yeast and mould species • High nutrient and temperature conditions could be modified to better represent environment • Could be utilised as a precursor assay to MSC testing to identify if resistant isolates are present – albeit more expensive than simple phenotypic AFST assays • Could potentially allow for testing of multiple antimicrobials and so would account for co-	• Doesn't consider mixing of antibiotics as exposed in the environment • Optimum laboratory conditions e.g., high temperatures and nutrient are not replicable of natural environment and may lead to possible under or overestimation of MSCs

			selection, which has been found to be of importance in environmental settings	
Efflux activity e.g., Ala-Nap and R6G	• Straightforward, quick and cost-effective • Direct measure of efflux useful in rapid identification of selective concentrations • Highlights specific mechanism responsible • Conventional methods well established	• Some dyes are transport system specific e.g., only applied to ABC transporters • Increase in replicates increases processing time – may compromise data quality and cost	• Provides efficient validation tool for overexpression or SELECT • Can be applied to both moulds and yeast • High nutrient and temperature conditions may be modified to better represent environment	• These assays will only detect resistance conferred by increase efflux • The testing of single antimicrobials does not account for co-selection, which has been found to be of importance in environmental settings • Optimum laboratory conditions e.g., high temperatures and nutrient are not replicable of natural environment and may lead to possible under or overestimation of MSCs • Would require identification of different targets to facilitate application to echinocandins and polyenes • Concern of high intrinsic resistance in many fungal species

				so drug-bug combinations must be case specific
Invertebrate host model e.g., *Galleria mellonella*	• Inexpensive, simple and allows greater replication • Used for a variety of fungal pathogens of clinical importance • Established protocol and thus reduces interlaboratory variability • Minimal ethics consideration for a host model organism • Provides data on both resistance and *in vivo* virulence	• Speculative, relationship between virulence and resistance not well studied • Does not provide information on resistance genes responsible	• Can be applied to both moulds and yeast • Could be applied to determine MSCs for polyenes and echinocandins	• Based on assumption decreased virulence confers for AMR, evidence to suggest otherwise – requires further investigation • Concern of high intrinsic resistance in many fungal species so drug-bug combinations must be case specific • The testing of single antimicrobials does not account for co-selection, which has been found to be of importance in environmental settings
Ergosterol content quantification **e.g., SQM**	• Inexpensive and simple, therefore allows greater replication • Ergosterol is universally important to all fungi, therefore this assay may be applied to a variety of species • Established protocols reduce interlaboratory variability of data	• Speculative, link between decreased ergosterol content and resistance not well documented • Does not provide information on resistance genes responsible • Limited to culturable microorganisms	• Potential as a standalone assay • Can be applied to both moulds and yeast • High nutrient and temperature conditions may be modified to better represent environment	• Based on assumption (though supported in comparison to AFST) that ergosterol content is a proxy for AFR • Cannot be used to determine selective effects of echinocandin resistance as ergosterol not involved

				in this drug class' mode of action • Optimum laboratory conditions e.g., high temperatures and nutrient are not replicable of natural environment and may lead to possible under or overestimation of MSCs • Concern of high intrinsic resistance in many fungal species so drug-bug combinations must be case specific • The testing of single antimicrobials does not account for co-selection, which has been found to be of importance in environmental settings

8. Concluding remarks

AFR is undoubtedly a growing, global human health problem. With high associated mortality rates and limited treatment alternatives available, AFR infections are a key concern. Furthermore, due to the incomplete removal or inactivation of antifungals during wastewater treatment and the direct application of effect antifungal concentrations to agricultural crops, the environmental dimension of AFR warrants greater exploration. Without considering resistance selection alongside traditional ecotoxicity endpoints or generating MSC data for antifungals of concern, the risk of AFR emergence in the environment is currently not effectively assessed.

The SELECT method, originally published by Murray and colleagues (2020), provides a unique and valuable tool to determine MSC values for antibiotics. Modifications of this assay and key considerations that may enable the use of this method for antifungals have been provided. These include the use of the assay specifically for yeast species and the initial use of haploid *C. auris* or *N. glabrata* with recommendations for future investigation into the use of additional species or community of fungi.

The SELECT method provides the cheapest and least labour-intensive experimental option and has the potential to be further adapted to provide crucial information on the evolution of AFR and identify environmental hotspots of AMR. It is advised that future research should prioritise the modification and validation of this method according to the specifications outlined here in line with traditional AFST broth microdilution protocols. To aid this, validation assays are proposed. In addition, given the number and diversity of fungal species of human health importance, including *A. fumigatus,* alternative assays are provided that may also be used to determine MSC data for complex mould species. This work may significantly improve our ability to inform release limits and risk assessment of antifungals.

8.1. Future study

Throughout this report, the current understanding of environmental antifungal sources, occurrence and fate is summarised. The synthesis of this information allowed us to propose modifications to the SELECT method (Murray et al., 2020), potential validation methods and possible standalone assays. There are currently no empirical antifungal MSC data available, therefore this report may provide valuable methods to fill these data gaps. Based on our recommendations, future work should prioritise the testing and validation of an antifungal SELECT method, alongside necessary validation.

Additional areas that require further investigation have also been identified:

- Currently, fluconazole is the only EU Water Framework Directive Hazardous Substance Watch List compound with a selective concentration defined. However, this was theoretically determined and there are no empirical MSC data available for any antifungal. Therefore, a first priority should be generation of MSC data for all azole antifungals of concern through monitoring programmes, including the Watch List. Should the SELECT method be successful in determining such data, this tool could be applied to additional antifungal agents of interest and may help to inform future substances of concern.

- Both the original and proposed antifungal SELECT methods seek to quantify selective endpoints of single antimicrobial agents. In the natural environment, microorganisms may be exposed to a complex mixture of antimicrobials and other pollutants, which can drive co-selection of resistance to multiple compounds. Therefore, future work should explore modifications to the SELECT, and other methods, to determine MSC data where inocula are exposed to a mixture of antimicrobials.

- WWTPs are important sources of antifungals and AFR fungi into the environment. However, the role of WWTPs in the evolution and dissemination of AFR in surface waters is not well understood. The antifungal SELECT method may provide a valuable tool to aid the evaluation of the role of WWTPs in environmental AFR, and could be further adapted to allow the identification of environmental hotspots of AFR.

- In addition to the previous point, the role of biosolid amended soils/sewage sludge in increasing exposure to residual antifungal contaminants is not yet understood. This is of additional importance, given that fungal soil communities have been previously found to harbour drug resistant human pathogens, including RAF. Assays to detect effect concentrations of antifungals against soil-borne fungi have been described here, with regard to *A. fumigatus*. However, soils are an important ecosystem for many fungal species and therefore require further consideration.

- Given that azoles are the only antifungal class applied both in clinical and agricultural settings, this report has predominantly focussed on these compounds. Though there is little evidence exploring the role of the environment in emergence of echinocandin or polyene resistance, future research should consider the monitoring of additional antifungal classes. This is especially important due to the lack of antifungal treatment options available, in order to effectively monitor the evolution and emergence of novel resistance in environmental fungal communities. Again, the SELECT method may provide a valuable and promising tool to address this.

- Finally, here the use of haploid yeast species to aid initial modifications and application of the SELECT method to test antifungals is recommended. However, future work should aim to consider diploid species, such as leading human fungal pathogen *C. albicans*. A systematic study using the validation tools outlined in Section 5 may be useful in comparing haploid and diploid selective endpoints, with the potential to reveal the importance of ploidy driven variability in selective effect concentrations. This would have further value in informing the future use of the antifungal SELECT method and highlight which species provides the least variable and representative MSC values.

9. Acknowledgements

Thank you to Alwyn Hart for suggesting application of bacterial methods to the study of AFR and Helen Wilkinson and Wiebke Schmidt at the Environment Agency for commissioning the project and for your guidance, ideas, support and extensive feedback throughout the project.

Thank you to the University of Exeter's AMR network (http://www.exeter.ac.uk/amr/) and MRC Centre for Medical Mycology (https://www.exeter.ac.uk/medicalmycology/) for support, scientific guidance and advice throughout the project.

This work was completed with thanks to Natural Environment Research Council Knowledge Exchange Fellowships NE/S006257/.

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List of abbreviations

Abbreviation	Definition
ABC	ATP Binding Cassette
AF	Assessment factor
AFR	Antifungal resistance
AFST	Antifungal susceptibility testing
Ala-Nap	Alanine-β-naphthylamide
AMR	Antimicrobial resistance
ARB	Antibiotic resistant bacteria
ARG(s)	Antibiotic resistance gene(s)
ASRIT	Activated sludge respiration inhibition test
ATP	Adenosine triphosphate
CAC	Codex Alimentarius Commission
cfu	Colony forming unit
CLSI	Clinical and Laboratory Standard Institute
DNA	Deoxyribonucleic Acid
ERA	Environmental risk assessment
ESBL	Extended spectrum beta-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing

FI(s)	Faecal indicator(s)
FIO(s)	Faecal indicator organism(s)
HGT	Horizontal gene transfer
HRM	High-resolution melt
IA	Invasive Aspergillosis
LD	Lanosterol demethylase
MDR	Multidrug resistance
MEC	Measured environmental concentration
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MIC_{susc}	MIC of susceptible bacteria
MoA	Mode of action
mRNA	Messenger ribonucleic acid
MSC	Minimal Selective Concentration
NOEC	No observed effect concentration
NWP	North West Province
OD	Optical density
PCR	Polymerase chain reaction
PEC	Predicted effect concentration
PNEC	Predicted no effect concentration

$PNEC^R$	Predicted no effect concentration for resistance
$PNEC^R_{MIC}$	MIC derived $PNEC^R$
PPP	Plant protection products
qPCR	Quantitative polymerase chain reaction
R6G	Rhodamine 6G
RAF	Resistant *Aspergillus fumigatus*
RQ	Risk quotient
RSM	Restriction site mutation
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SDA	Sabouraud's dextrose agar
SDBM	Sabouraud's dextrose broth medium
SELECT	Selection endpoints in communities of bacteria
SN	Surveyor nuclease
SNP(s)	Single nucleotide polymorphism(s)
SQM	Sterol quantitation method
T-ARMS-PCR	Tetra primer-amplification refractory mutation system-PCR
TR	Tandem repeat
TR_{34}/L98H	TR sequence of 34 base pairs with L98H mutations
TR_{46}/Y121F/T286A	TR sequence of 46 base pairs with Y121F/T289A mutations
WFD	Water Framework Directive

WGS	Whole genome sequencing
WL	Watch List
WWTP(s)	Wastewater treatment plant(s)

Appendix A: environmental concentrations of azole antifungals

Appendix A. Environmental concentrations of key (listed on 3rd EU Watch List) azole antifungals (extracted from Bhagat et al., 2020; Chen and Ying, 2015).

Azole compound	Country	Surface water (ng/L)	Wastewater effluent (ng/L	Reference
Clotrimazole	UK	n.d. - 22		Thomas and Hilton (2004)
	UK	9-27.3	10-27	Roberts and Thomas (2006)
	UK	6	34	Peschka et al. (2007)
	Switzerland		n.d.-6	Kahle et al. (2008)
	China	4	8	Huang et al. (2010)
	China	n.d.	2.7	Chen et al. (2012)
	Poland	0.7-29	5.9-31.1	Zgoła-Grześkowiak and Grześkowiak (2013)
	Spain	7-9	5-11	Casado et al. (2014)
	China	n.d. – 0.48		Liu et al. (2015)
	China	0.68–102.98		Zhang et al. (2015)
	South Africa		30.03	Assress et al. (2019)
Fluconazole	Switzerland	n.d. -9	28-83	Kahle et al. (2008)
	China	2	3	Huang et al. (2010)
	China		50-139	Peng et al. (2012)
	China	n.d.	61.1	Chen et al. (2012)
	China	133	85-448	Chen et al. (2014)
	Spain	25-32	37-95	Casado et al. (2014)
	China	1.15-28.4		Liu et al. (2015)
	China	2.81–13.6		Zhang et al. (2015)
	South Africa		302.38	Assress et al. (2019)
Miconazole	UK	7.5	9	Roberts and Bersuder (2006)
	China	3	2	(Huang et al. (2010)
	Belgium		35.7	Van De Steene et al. (2010)
	China	n.d.	0.5	Chen et al. (2012)
	China	8.34	0.5-9	Huang et al. (2013)
	China	n.d. - 30.7	0.89-4.6	Chen et al. (2014)
	Spain	5	11-15	Casado et al. (2014)
	China	0.10-0.35		Liu et al. (2015)
Tebuconazole	Switzerland	n.d. -1	10	Kahle et al. (2008)
	Germany	2.4-5.9	3.6-6.4	Wick et al. (2010)
	Spain	1.66-15.38		Ccanccapa et al. (2016)
	China	32.58		Peng et al. (2018)

n.d.: Not detected.

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