



Environmental Drivers of Azole-Resistance in *Aspergillus fumigatus* and its Relevance to Human Health

Jennifer May Grace Shelton

Department of Infectious Disease Epidemiology
Imperial College London
CID: 00697052

Supervisors:

Professor Matthew Fisher and Dr Andrew Singer

A thesis submitted for the degree of Doctor of Philosophy

July 2021

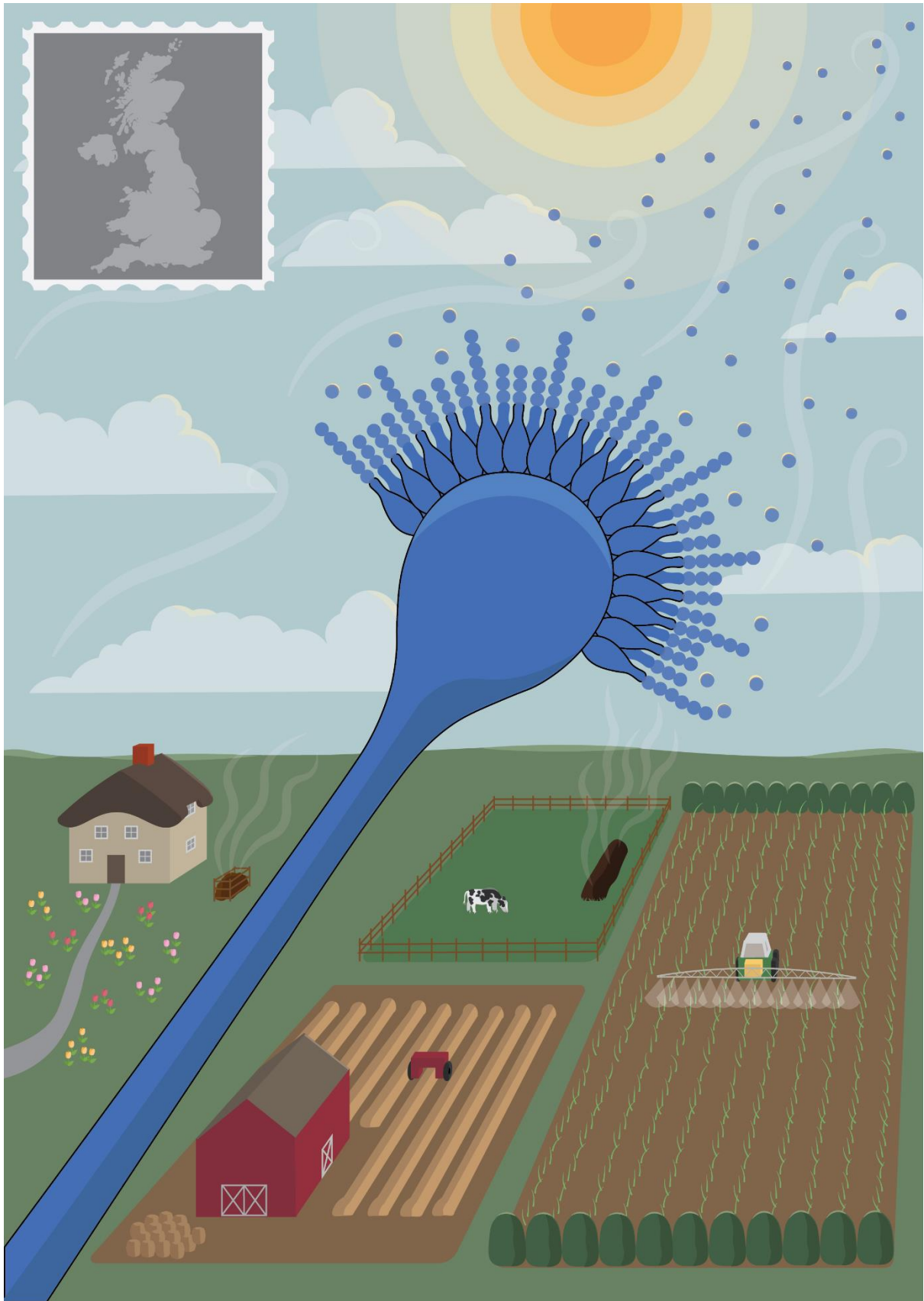


Image designed by Jennifer Shelton and produced by 'Designs that Cell'.

Declaration of Originality

I declare that the work presented in this thesis is original and my own, and has been approved by my supervisors. Input from contributors has been acknowledged in every instance.

Copyright Declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-NonCommercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Acknowledgements

Thank you to NERC for funding my project through the Grantham Institute for Climate Change and the Environment's Science and Solutions for a Changing Planet Doctoral Training Program.

Thank you to Mat and Andrew for providing the perfect combination of supervision, project, groups and workplaces to make my PhD an enjoyable and valuable experience – I hope to do you both justice with my future work. To my rock Matt – thank you for not baulking when I said I wanted to study again and for supporting me throughout my PhD, and always. I hope you can find something you love to do as much as I've loved my PhD! To my mum – thank you for being my best friend and number one supporter, and for instilling in me such a passion for learning that is still going strong 30 years later. To my dad – thank you for your support and for introducing me to sport; fitness kept me sane and motivated when the world turned upside down last year! To my amazing friends Claire and Hayley – I don't know what I would have done without you two, especially in the past 18 months, so thank you for always being there for me. To my D&D group – thank you for the much-needed escapism over the past four years, and especially during lockdown when we couldn't leave the house! To my grandad – thank you also for making me passionate about learning and I hope to celebrate my thesis hand-in with you. Finally, not that he can read this, but extra treats for our cat Smithy who has sat on a cushion beside me and kept me company for the whole of lockdown working-from-home.

Thank you to my Imperial College London colleagues Jo, Pria, Thomas, Amelie, Sam, Kieran, Phil, Hannah, Simon and Susan for creating a fun and supportive work environment during my PhD and helping me out when I couldn't get into London. Thank you to my UKCEH colleagues Ellie, Holly, Elmer, Issy, Lea, Jordan, Doug, Katie and Dom for the lab chats, tea breaks and pub trips – I miss working in an office with you.

Contributions to thesis content are acknowledged at the ends of Chapters 3, 4, 5 and 6.

Abstract

Aspergillus fumigatus is a fungus that plays an important role in nature and compost production by recycling nutrients from decaying organic matter. The spores are found everywhere – in air and in soil, indoors and outdoors and on every continent except Antarctica - and it is estimated that humans inhale several hundred spores every day. Due to their small size and thermotolerance, *A. fumigatus* spores are able to enter lung cavities where they can establish and cause infections if not cleared by the innate immune system. It was estimated that in 2011 there were as many as 250,000 individuals in the UK suffering from aspergillosis, with diseases ranging from severe allergic reaction to spore inhalation to invasive infections when spores migrate from the lung into the bloodstream. Aspergillosis is treated with drugs containing azoles, and the proportion of azole-resistant infections in the UK has increased from 3-7% between 1999 and 2001 to 14-20% between 2007 and 2009. Azole-resistant infections result in treatment failure, salvage therapy with more toxic antifungals and decreased patient survival. *A. fumigatus* acquires azole-resistance in the patient after long-term treatment with azoles but azole-resistant *A. fumigatus* is also detected in patients who have never been treated with azoles, implying that the infecting spore had acquired resistance before it was inhaled. This is most likely due to adaptation of *A. fumigatus* in the environment to azoles found in agricultural fungicides with similar structures and modes of action to medical azoles. This thesis seeks to understand the distribution of azole-resistant *A. fumigatus* in the UK and the environmental factors that may lead to its adaptation through natural selection. To do this, a citizen science approach to sample collection was used; with volunteers in England, Wales, Scotland and Northern Ireland collecting air and soil samples from which *A. fumigatus* was cultured.

Table of Contents

Declaration of Originality	3
Copyright Declaration	3
Acknowledgements.....	4
Abstract.....	5
Table of Contents.....	6
List of Tables	13
List of Figures	16
Acronyms and Abbreviations	19
Chapter 1: Fungi as Pathogens	22
1.1. Plant, animal and human pathogens	23
1.1.1. Plant pathogens	23
1.1.2. Animal pathogens	25
1.1.3. Human pathogens.....	27
1.2. <i>Aspergillus fumigatus</i> and aspergillosis	29
1.2.1. Dispersal of <i>A. fumigatus</i> in the air.....	29
1.2.2. <i>A. fumigatus</i> in the soil	30
1.2.3. <i>A. fumigatus</i> as a human pathogen	30
1.3. Drug-resistant <i>A. fumigatus</i>	32
1.3.1. During infection	32
1.3.2. In the environment	33
1.3.3. Mechanisms of azole-resistance	34
1.3.4. Evidence for environmental route of azole-resistance development	35
1.3.5. The growing problem of azole-resistant <i>A. fumigatus</i>	36
1.4. Project aims.....	37
Chapter 2: General Methods	40
2.1. Safe handling of <i>Aspergillus fumigatus</i> in the laboratory.....	40
2.2. Preparation of Sabouraud dextrose agar plates for culturing <i>A. fumigatus</i>	40
2.3. Culturing <i>A. fumigatus</i> from air samples	42
2.4. Testing <i>A. fumigatus</i> isolates from air samples for tebuconazole-resistance	43
2.5. Culturing <i>A. fumigatus</i> from soil samples and testing for tebuconazole-resistance	44
2.6. Cryopreserving <i>A. fumigatus</i> isolates	44
2.7. Extracting DNA from <i>A. fumigatus</i> isolates.....	45
2.8. Testing <i>A. fumigatus</i> isolates for medical azole resistance	45
2.8.1. Media preparation	46

2.8.2.	Preparation of antifungal drug dilutions.....	46
2.8.3.	Preparation of microdilution plates.....	47
2.8.4.	Preparation of isolates for susceptibility testing	47
2.8.5.	Inoculation of microdilution plates.....	48
2.8.6.	Reading test results.....	48
2.9.	Sanger sequencing of <i>cyp51A</i> gene and promoter region.....	49
2.9.1.	Initial polymerase chain reaction.....	49
2.9.2.	Sanger sequencing	52
2.10.	Confirming identity of isolates as <i>A. fumigatus</i>	54
2.10.1.	Macroscopic appearance	54
2.10.2.	Microscopic appearance	55
2.10.3.	Matrix assisted laser desorption ionization-time of flight mass spectrometry	55
2.10.4.	Sanger sequencing of beta-tubulin gene	55
2.11.	Whole-genome sequencing of <i>A. fumigatus</i> isolates	56
2.11.1.	DNA extraction and whole-genome sequencing	56
2.11.2.	Bioinformatic analysis	56
2.11.3.	Phylogenetic analysis	57
2.12.	Maximum daily air temperatures on UK sampling dates	57
2.13.	Land cover classifications of UK sampling locations	58
2.14.	Percentage of arable land cover in 2 km surrounding UK sampling locations	59
2.15.	Distances from sampling locations to nearest composting facility with open windrow or outdoor activity.....	60
2.15.1.	In England.....	60
2.15.2.	In Scotland.....	61
2.15.3.	In Wales.....	61
2.15.4.	In Northern Ireland	62
2.16.	Generalised linear models	62
Chapter 3:	Air- and Soil-Sampling Mycology Citizen Science Projects.....	63
Abstract.....		63
3.1.	Introduction	64
3.1.1.	History of citizen science.....	64
3.1.2.	Ecological data collection by citizen scientists.....	64
3.1.3.	Recording invisible threats to species and environments	65
3.1.4.	Examples of citizen science in mycology	65
3.1.5.	Environmental sampling for azole-resistant <i>Aspergillus fumigatus</i>	66
3.2.	Methods.....	67

3.2.1.	Air sampling trial run.....	67
3.2.2.	Recruitment for citizen science air sampling project.....	68
3.2.2.1.	Initial air sampling round	68
3.2.2.2.	Subsequent air sampling rounds.....	69
3.2.3.	Participating in citizen science air sampling	69
3.2.4.	Recruitment for citizen science soil sampling project	70
3.2.5.	Participating in citizen science soil sampling	71
3.2.6.	Citizen science engagement.....	71
3.3.	Results.....	72
3.3.1.	Citizen science air sampling	72
3.3.1.1.	In the UK.....	72
3.3.1.2.	Globally	74
3.3.2.	My participation in air sampling	74
3.3.3.	Citizen science soil sampling.....	74
3.3.4.	Culturing <i>A. fumigatus</i> from samples	75
3.3.5.	Adherence to sampling date for UK samples.....	76
3.3.6.	Recruitment methods	77
3.3.7.	Participant engagement and feedback.....	77
3.3.8.	Amendments to sampling methodology	79
3.4.	Discussion.....	80
3.5.	Acknowledgements.....	84
Chapter 4:	Azole-Resistant <i>Aspergillus fumigatus</i> Spores in UK Air	85
	Abstract.....	85
4.1.	Introduction	86
4.1.1.	Airborne <i>Aspergillus fumigatus</i> spores.....	86
4.1.2.	Air sampling methodologies	87
4.1.2.1.	Culture-based.....	87
4.1.2.2.	PCR-based	87
4.1.3.	Air sampling for <i>A. fumigatus</i> in the UK.....	88
4.1.3.1.	Industrial composters	88
4.1.3.2.	Hospitals.....	89
4.1.3.3.	Background levels	89
4.1.4.	Seasonal differences in levels of airborne <i>A. fumigatus</i> spores	90
4.1.5.	Background levels of azole-resistant airborne <i>A. fumigatus</i> spores.....	91
4.2.	Methods.....	92
4.2.1.	Air sampling methodology	92

4.2.2.	Processing air samplers, culturing <i>A. fumigatus</i> and testing for ARAf	93
4.2.3.	Cross-resistance to medical azoles	93
4.2.4.	Cryopreservation, DNA extraction and gene sequencing of ARAf.....	94
4.2.5.	Confirming identity as <i>A. fumigatus</i>	94
4.2.6.	Whole-genome sequencing	94
4.2.7.	Environmental factors relating to UK sampling dates and locations.....	94
4.2.8.	Generalised linear models	95
4.2.9.	Geographically weighted regression.....	96
4.3.	Results.....	97
4.3.1.	Validation of air sampling methodology.....	97
4.3.2.	ARAf in UK air samples.....	97
4.3.2.1.	Cross-resistance of UK tebuconazole-resistant airborne <i>A. fumigatus</i> to medical azoles	100
4.3.2.2.	Confirming identity of UK airborne ARAf.....	102
4.3.2.3.	<i>Cyp51A</i> polymorphisms in UK airborne ARAf	103
4.3.3.	ARAf in global air samples.....	103
4.3.3.1.	Cross-resistance of global tebuconazole-resistant airborne <i>A. fumigatus</i> to medical azoles.....	103
4.3.3.2.	Confirming identity of global airborne ARAf.....	104
4.3.3.3.	<i>Cyp51A</i> polymorphisms in global airborne ARAf.....	104
4.3.4.	Whole-genome sequencing of SS18 ARAf isolates	104
4.3.5.	Maximum air temperatures on air sampling dates	105
4.3.6.	Land cover classifications of UK sampling locations	106
4.3.7.	Land cover classifications of composting facilities with open windrow or outdoor activity in the UK.....	108
4.3.8.	Distances from sampling locations to nearest composting facility	109
4.3.9.	Percentage of arable land in 2 km buffer surrounding sampling locations.....	110
4.3.10.	Factors influencing whether samples grew <i>A. fumigatus</i>	111
4.3.10.1.	Logistic regression model for UK samples	111
4.3.10.2.	Geographical weighted regression models for UK samples	114
4.3.11.	Factors influencing the number of <i>A. fumigatus</i> colonies grown from a sample.....	117
4.3.11.1.	Negative binomial model for UK samples.....	117
4.3.12.	Factors influencing whether samples grew ARAf	118
4.3.12.1.	Logistic regression model for UK samples	118
4.3.13.	Factors influencing the number of ARAf colonies grown from a sample	118
4.3.13.1.	Negative binomial model for UK samples.....	118

4.4.	Discussion.....	118
4.5.	Acknowledgements.....	124
Chapter 5:	Azole-Resistant <i>Aspergillus fumigatus</i> Spores in UK Soil.....	125
	Abstract.....	125
5.1.	Introduction	126
5.1.1	Land use in the UK	126
5.1.1.1.	Composting practices in the UK.....	127
5.1.1.2.	Occupational risk of chronic aspergillosis.....	128
5.1.1.3.	Compost and gardening in the UK	129
5.1.1.4.	Aspergillosis from gardening.....	129
5.1.1.5.	<i>A. fumigatus</i> and azole-resistant <i>A. fumigatus</i> in UK soils	130
5.2.	Methods.....	133
5.2.1.	Recruitment for citizen science sampling project.....	133
5.2.2.	Processing soil samples, culturing <i>A. fumigatus</i> and testing for ARAf.....	133
5.2.3.	Cryopreservation, DNA extraction and gene sequencing of ARAf.....	134
5.2.4.	Confirming identity as <i>A. fumigatus</i>	134
5.2.5.	Environmental factors relating to UK sampling dates and locations.....	134
5.2.6.	Generalised linear models	135
5.3.	Results.....	136
5.3.1.	<i>A. fumigatus</i> and ARAf in UK soil samples	136
5.3.2.	<i>Cyp51A</i> polymorphisms in UK soilborne ARAf.....	138
5.3.3.	Maximum air temperatures on soil sampling dates	139
5.3.4.	Land cover classifications of sampling locations.....	140
5.3.5.	Land cover classifications of composting facilities with open windrow or outdoor activity in the UK.....	142
5.3.6.	Distance from UK sampling locations to nearest composting facility	142
5.3.7.	Percentage of arable land in 2km buffer surrounding sampling locations.....	143
5.3.8.	Factors influencing whether samples grew <i>A. fumigatus</i>	144
5.3.8.1.	Logistic regression model for UK samples	144
5.3.9.	Factors influencing the number of <i>A. fumigatus</i> colonies grown from a sample.....	145
5.3.9.1.	Negative binomial model for UK samples.....	145
5.3.10.	Factors influencing whether samples grew ARAf	146
5.3.10.1.	Logistic regression model for UK samples	146
5.3.11.	Factors influencing the number of ARAf colonies grown from a sample	148
5.3.11.1.	Negative binomial model for UK samples.....	148

5.3.12.	Growth of <i>A. fumigatus</i> and ARAf colonies from air and soil sampled from the same location	150
5.4.	Discussion.....	150
5.5.	Acknowledgements.....	155
Chapter 6:	Is Climate Change a Concern for Azole-Resistant <i>Aspergillus fumigatus</i> ?	156
	Abstract.....	156
6.1.	Introduction	157
6.1.1.	Climate change and infectious diseases	157
6.1.2.	Climate change and fungal pathogens.....	158
6.1.2.1.	Plant fungal pathogens	158
6.1.2.2.	Human fungal pathogens.....	159
6.1.3.	Climate change and antifungal resistance	160
6.1.4.	Hypothesised role of ergosterol in thermal adaptation of <i>Aspergillus fumigatus</i>	160
6.1.5.	Potential evidence for thermal adaptation of <i>A. fumigatus</i> in this thesis.....	161
6.2.	Methods.....	162
6.2.1.	Discovery of loss of azole-resistance in <i>A. fumigatus</i> isolates grown from air samples	162
6.2.2.	Discovery of loss of azole-resistance in <i>A. fumigatus</i> isolates grown from soil samples	163
6.2.3.	Sanger sequencing of 1° and 2° isolates	164
6.2.4.	Whole-genome sequencing of SS18 1° and 2° isolates.....	164
6.2.5.	Temperature experiment for loss or gain of azole-resistance in <i>A. fumigatus</i> isolates	164
6.3.	Results.....	165
6.3.1.	<i>A. fumigatus</i> isolates grown from air samples that lost azole-resistance	165
6.3.2.	Whole-genome sequencing of SS18 1° and 2° isolates.....	167
6.3.3.	<i>A. fumigatus</i> isolates grown from soil samples that lost azole-resistance.....	168
6.3.4.	<i>A. fumigatus</i> isolates that lost or gained azole-resistance during the temperature experiment.....	169
6.4.	Discussion.....	170
6.5.	Acknowledgements.....	174
Chapter 7:	General Discussion	176
7.1.	Key findings.....	177
7.2.	Future directions.....	180
References		183
Appendices.....		203

Appendix 1: Infographic of thesis including samples collected, consumables used, sequencing undertaken, places travelled to and people involved.	203
Appendix 2: Importance of fungi in nature, food, medicine, industry and bioremediation. .	204
Appendix 3: Google sign-up form for the first air sampling round that was linked to by a shortened URL on the poster and in all recruitment Tweets, Facebook posts and emails. ...	218
Appendix 4: Instructions explaining when and how to expose the air samplers to participate in the citizen science air sampling project. These instructions were for initial air sampling round on 21 st June 2018 and the date was amended for subsequent sampling rounds.	220
Appendix 5: Questionnaire to be completed and returned in the Freepost envelope detailing the date air samples were collected and the exact locations where the air samplers were placed and exposed. Participants were asked to provide an email address if they wished to receive future project updates.	221
Appendix 6: A blog post written about the air sampling citizen science project was published online when recruitment began, and a paper copy was included in each sampling pack.	222
Appendix 7: Blog post about soil sampling citizen science project published on UKCEH website.....	224
Appendix 8: Instructions explaining how to collect soil samples to participate in the citizen science experiment.	227
Appendix 9: Questionnaire to be completed for the soil sampling project and returned in the Freepost envelope detailing the date sample collected, geographical location of the garden that soils were collected from, type of sample collected and sample details.....	228
Appendix 10: Google form sent out four days after the first air sampling round asking participants for feedback on their sampling experience.	229
Appendix 11: Participant feedback on the first air sampling round provided in the additional comments box of the Google form sent out on 25 th June 2018.	231
Appendix 12: Timeline of changes made to air sampling methodology across the four air sampling rounds as a result of participant feedback.....	235
Appendix 13: Maximum air temperatures for dates that air samples were collected, according to Met Office daily climate data for sample location.	236
Appendix 14: Details of 736 <i>Aspergillus fumigatus</i> isolates cultured from soil samples that grew at tebuconazole 6 mg/L; including the sample they were grown from, the county of the sampling location, the garden location from which the sample was collected and the <i>cyp51A</i> polymorphisms that were detected.	237
Appendix 15: Certificate of Attendance for Public Health England Mycology Reference Laboratory, Bristol.	242
Appendix 16: Certification for participation in Young Entrepreneur Scheme (YES) 2020 with business proposal “TerraGrow” – mycorrhizal mixes of fungi for promoting growth of newly-planted trees.....	243
Appendix 17: Co-authored papers published during PhD that are relevant to this thesis.....	244
Appendix 18: Blogs and articles published during PhD that are relevant to this thesis.....	245

List of Tables

Table 1.1: An overview of the chapters in this thesis and a brief description of their contents.	38
Table 2.1: Details of dilutions to be carried out to ascertain required concentrations of drugs in containers 1 to 10, starting from a 320 mg/L stock solution in RPMI medium.	46
Table 2.2: Details of the primers, master mix solutions and PCR conditions used to amplify the promoter and coding regions of <i>cyp51A</i>	50
Table 2.3: Land cover classifications 1-21 and descriptions for UK Centre for Ecology & Hydrology's land cover map 2019.	58
Table 3.1: Numbers of sampling packs sent out and number of packs and samples returned across the four air sampling rounds and one soil sampling round.	72
Table 3.2: Numbers of samples that grew <i>A. fumigatus</i> colonies and the number of colonies cultured across the four air sampling rounds and one soil sampling round. ^a These numbers represent fungal isolates cultured from samples that were identified macroscopically as <i>A. fumigatus</i>	76
Table 3.3: Dates that UK samples were collected for four air sampling rounds and one soil sampling round.	76
Table 4.1: Dependent and independent variables used in generalised linear model fitting to determine the influence of climate and location on growth of <i>A. fumigatus</i> and azole-resistant <i>A. fumigatus</i> . ^a Counts of 0 were excluded to avoid replicating the logistic regressions. ^b Data available for samples collected in England, Wales, Scotland and Northern Ireland hence samples collected from the Republic of Ireland were not included. OW/OA composter = composter with open windrow or outdoor activity.	95
Table 4.2: The number of air samples collected, the number of air samples that grew <i>A. fumigatus</i> and the number of samples that grew azole-resistant <i>A. fumigatus</i> across the four air sampling dates.	98
Table 4.3: The number of <i>A. fumigatus</i> and azole-resistant <i>A. fumigatus</i> isolates grown from air samples and the average number of <i>A. fumigatus</i> isolates grown per sample across the four air sampling dates.	98
Table 4.4: Details of the 111 UK tebuconazole-resistant isolates including the sampling round they were collected in, the unique identifier for the air sample they were grown from, the county from which the sample was collected, their minimum inhibitory concentrations (MICs) for itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ), and their <i>cyp51A</i> polymorphisms. Resistant MICs are highlighted in bold and isolates subsequently found not to be <i>A. fumigatus</i> are highlighted in red. WT=wild-type. ND=not done.	102
Table 4.5: Details of the nine global tebuconazole-resistant isolates including the sampling round they were collected in, the unique identifier for the air sample they were grown from, the country from which the sample was collected, their minimum inhibitory concentrations (MICs) for itraconazole (ICZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ), and their <i>cyp51A</i> polymorphisms. Resistant MICs are highlighted in bold and isolates subsequently found not to be <i>A. fumigatus</i> are highlighted in red.	104
Table 4.6: Breakdown of land cover classes for air sampling locations by country, obtained from UK Centre for Ecology & Hydrology's 2019 land cover maps for Great Britain and Northern Ireland. ...	108
Table 4.7: Breakdown of land cover classifications for locations of composting facilities in the UK with open windrow or outdoor activity by country. Land cover data were obtained from UK Centre for Ecology & Hydrology's 2019 land cover maps for Great Britain and Northern Ireland.	109
Table 4.8: Breakdown by country of the percentage of arable land within surrounding 2 km of unique sampling locations.	110

Table 4.9: Odds ratios, confidence intervals and p-values calculated for independent variables included in a logistic regression model using samples collected in the UK ($n = 1,889$) to explain whether a sample grew <i>A. fumigatus</i> . Significant results ($p \leq 0.05$) are highlighted in bold.	
^a Insufficient data to calculate odds ratio and confidence intervals.	112
Table 4.10: Summary of geographically weighted regression outputs for UK samples, run separately for each sampling round. ^a GLM refers to the non-spatial logistic regression model with growth of <i>A. fumigatus</i> as the binary outcome.	114
Table 5.1: Dependent and independent variables used in generalised linear model fitting to determine the influence of climate and location on growth of <i>A. fumigatus</i> and azole-resistant <i>A. fumigatus</i> . ^a It was unknown for eight samples whether <i>A. fumigatus</i> grew or not due to contamination. ^b Counts of 0 were excluded to avoid replicating the logistic regressions. OW/OA composter = composter with open windrow or outdoor activity.	135
Table 5.2: A breakdown of the number of soil samples collected, the number and percentage of soil samples that grew <i>A. fumigatus</i> and the number and percentage of samples that grew azole-resistant <i>A. fumigatus</i> (ARAF) by the location(s) in the garden that the soil sample was collected from.	137
Table 5.3: Numbers of <i>A. fumigatus</i> and azole-resistant <i>A. fumigatus</i> (ARAF) isolates grown from each garden location that soil samples were collected from, and the average colony-forming units per gram (CFU/g) for each location. ^a Calculated as the number of <i>A. fumigatus</i> isolates grown divided by the number of samples that grew <i>A. fumigatus</i> , multiplied by 20 to account for dilution factor of soil in buffer.	137
Table 5.4: The number of <i>A. fumigatus</i> and non- <i>A. fumigatus</i> isolates (failed to sequence) grown from soils collected from each garden location that were azole-resistant, and a summary of their <i>cyp51A</i> polymorphisms. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.	139
Table 5.5: Breakdown of land cover classifications for soil sampling locations by country, obtained from UK Centre for Ecology & Hydrology's 2019 land cover maps for Great Britain and Northern Ireland.	142
Table 5.6: Breakdown by country of the percentage of arable land within surrounding 2 km of unique sampling locations.	143
Table 5.7: Odds ratios, confidence intervals and p-values calculated for independent variables included in a logistic regression model using samples collected in the UK ($n = 501$) to explain whether a sample grew <i>A. fumigatus</i> . Significant results ($p \leq 0.05$) are highlighted in bold. ^a Insufficient data to calculate odds ratio and confidence intervals. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.	145
Table 5.8: Odds ratios, confidence intervals and p-values calculated for independent variables included in a logistic regression model using samples collected in the UK ($n = 509$) to explain whether a sample grew azole-resistant <i>A. fumigatus</i> . Significant results ($p \leq 0.05$) are highlighted in bold. ^a Insufficient data to calculate odds ratio and confidence intervals. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.	147
Table 5.9: Details of 46 UK locations from which both an air and soil sample were collected, including the county and the number of <i>A. fumigatus</i> (A) and azole-resistant <i>A. fumigatus</i> (R) colonies grown from each sample. Samples that grew azole-resistant <i>A. fumigatus</i> are highlighted in red and locations that grew azole-resistant <i>A. fumigatus</i> from both air and soil samples are highlighted in bold.	149
Table 6.1: Overview of experimental setup for storage of <i>A. fumigatus</i> isolates in three different media and at three different temperatures over 18 weeks to determine whether any isolates lost or	

gained resistance to tebuconazole at 6 mg/L. RT=room temperature, MPS=mould preservation solution, diH ₂ O=deionised water.	165
Table 6.2: The unique identifiers of the 14 tebuconazole-resistant isolates that lost tebuconazole-resistance after refrigeration in mould preservation solution. The minimum inhibitory concentrations (MICs) for itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ) and <i>cyp51A</i> polymorphisms are given for the primary (1 ^o) and secondary (2 ^o) isolates. All isolates were collected in the summer air sampling round, denoted by the prefix SS18. WT=wild-type. Resistant MICs are highlighted in bold.	166
Table 6.3: The number of samples that grew azole-resistant isolates and the average time in months between the first and second tebuconazole susceptibility test on isolates, for each air sampling round.....	166
Table 6.4: Pairwise genetic distances calculated by RAxML, SNP differences and relatedness rank out of 28,203 for 13 pairs of primary (1 ^o) and secondary (2 ^o) SS18 isolates.	168
Table 6.5: The unique identifiers of the 28 tebuconazole-resistant isolates that lost tebuconazole-resistance after refrigeration in mould preservation solution. The <i>cyp51A</i> polymorphisms are given for the primary (1 ^o) and secondary (2 ^o) isolates. All isolates were collected in the soil sampling round, denoted by the prefix SS19. WT=wild-type.	169
Table 6.6: Five isolates grown from garden air samples that were susceptible to tebuconazole on day 0 of the experiment but exhibited full (+) or partial (o) growth in the presence of tebuconazole at a later date in the experiment. The storage medium and temperature are detailed for each isolate. RT=room temperature.	170
Table 6.7: <i>Cyp51A</i> polymorphisms for isolates at week 0 and at the first week that the isolate grew on tebuconazole for the combination of storage medium and temperature. ^a JG7 was not sequenced again after day 0 as it maintained tebuconazole-resistance throughout the experiment.....	170

List of Figures

Figure 1.1 Timeline of dates for the global introduction of azole compounds into agriculture (shown in green) and medicine (shown in blue). The most-used agricultural azoles globally in 2008 are highlighted in bold and first reports of azole resistance are shown in red.	32
Figure 1.2 Barplot showing the number of patient cases with <i>A. fumigatus</i> infection in the UK between 1997 and 2009 and the percentage of patients with an azole-resistant infection, adapted from Bueid <i>et al.</i> (2010) ¹⁰¹ . ITZ=itraconazole, PCZ=posaconazole, VCZ=voriconazole.	33
Figure 2.1: Plates and Nunc™ flasks left to dry in the hood for an hour once filled with Sabouraud dextrose agar.	42
Figure 2.2: A) Itraconazole, posaconazole, voriconazole and isavuconazole drug plates filled with eight isolates ready for incubation, and B) a drug plate after incubation ready for isolate minimum inhibitory concentrations to be read and recorded.	48
Figure 2.3: Illustration of <i>cyp51A</i> gene showing tandem repeats in the promoter region and amino acid substitutions caused by point mutations in the coding region, adapted from Zhang <i>et al.</i> (2017) ¹³⁵	49
Figure 2.4: Electrophoresis gel images showing A) amplicons from <i>cyp51A</i> coding region PCR (left) and <i>cyp51A</i> promoter region PCR (right) for the same seven DNA samples, B) a 96-well plate of samples from promoter region PCR where all samples have amplified except for the negative control, and C) a 96-well plate of samples from promoter region PCR where several samples have not amplified.	51
Figure 2.5: DNA sequence of <i>cyp51A</i> promoter and coding regions, with primers used to amplify and sequence the promoter region highlighted in turquoise and primers used to amplify and sequence the coding region highlighted in yellow. The insert point for the 34 base pair tandem repeat (TR ₃₄) and the single nucleotide polymorphism that results in the L98H amino acid substitution are shown for reference.	52
Figure 2.6: A) DNA alignment of the <i>cyp51A</i> promoter region for 20 samples with their wild-type, TR ₃₄ or TR ₄₆ status annotated on the right-hand side, and B) protein alignment for nine samples translated from a DNA alignment, with L98H amino acid substitutions annotated on the right-hand side.	53
Figure 2.7: A) Macroscopic appearance of <i>A. fumigatus</i> as blue green colonies after 48 hours of growth on Sabouraud dextrose agar at 43°C, and B) microscopic appearance of <i>A. fumigatus</i> spore heads at 400X magnification.	54
Figure 2.8: Arable land cover in A) Northern Ireland and B) Great Britain shown here in black after land cover class 3 was extracted from the UKCEH 2019 land cover maps.	60
Figure 2.9: A) Map showing the location of composting facilities in the UK with open windrow or outdoor activity: green dots are sites in England, pink in Wales, yellow in Scotland and purple in Northern Ireland, and B) screenshot from Google maps showing an open windrow composting facility.	61
Figure 3.1: A trial run of the air sampling method was undertaken at A) St Mary's campus of Imperial College London, Paddington for two, four and six hours, and B) UKCEH Wallingford, Oxfordshire for eight hours.	68
Figure 3.2: Posters advertising the four rounds of the air sampling citizen science project (left panel of four) and soil sampling project (right panel).	70
Figure 3.3: Google maps showing locations that participants collected air samples from on A) 21 st June 2018, B) 24 th September 2018, C) 21 st December 2018 and D) 20 th March 2019. E) shows locations that soil samples were collected from on 21 st June 2019. Blue dots indicate samplers that	

did not grow <i>A. fumigatus</i> colonies, green dots indicate samplers that did and red dots indicate samplers that were contaminated with other fungal growth. F) is a population density map of the UK adapted from Vieno <i>et al.</i> (2015) ¹⁹⁴	73
Figure 3.4: Photos of me participating in air sampling on A) 21 st June 2018, B) 24 th September 2018, C) 21 st December 2018 and D) 20 th March 2019. E) is a screenshot of a Google map showing the route I drove around Oxfordshire and the Cotswolds on each of the four air sampling dates.....	75
Figure 3.5: Several Tweets posted by participants on sampling days showing them taking part in air and soil sampling. (Permission granted by participants for these Tweets to be displayed.)	78
Figure 3.6: A) Air samplers for first air sampling round had sticky labels on the back with location number and basic instructions, but several participants peeled this off instead of the back of the air sampler. B) For subsequent air sampling rounds, location number was handwritten on the back of each air samplers to avoid confusion on how to expose the air sampler.	80
Figure 4.1: <i>Aspergillus/Penicillium</i> spore concentrations in outdoor air samples collected in Derby by the Midlands Asthma & Allergy Research Association (MAARA) from 1991 to 2003. Produced from data reported in Millington <i>et al.</i> (2005) ²²⁵	90
Figure 4.2: Histograms showing the distribution of numbers of A) <i>A. fumigatus</i> colonies and B) azole-resistant <i>A. fumigatus</i> colonies grown from positive samples.	96
Figure 4.3: Maps showing locations of UK and Republic of Ireland air samples collected on A) 21 st June 2018, B) 24 th September 2018, C) 21 st December 2018 and D) 20 th March 2019. Blue dots indicate samples that did not grow <i>A. fumigatus</i> colonies, green dots indicate samples that did and red dots indicate samples that grew azole-resistant <i>A. fumigatus</i>	99
Figure 4.4: Bootstrapped phylogeny of 212 clinical and environmental <i>A. fumigatus</i> isolates collected from across the UK between 2005 and 2017 ²³¹ , 37 tebuconazole-resistant <i>A. fumigatus</i> isolates collected from the UK in the first air sampling round (prefix SS18) and eight tebuconazole-resistant <i>A. fumigatus</i> isolates collected globally in the first air sampling round (prefix SS18). UK SS18 isolates are denoted by red lines and global SS18 isolates by green lines.	105
Figure 4.5: Boxplot showing the maximum daily temperatures at the locations that samples were collected from for each sampling round.....	106
Figure 4.6: The 803 unique locations in the UK and Republic of Ireland that air samples were collected from across the four sampling rounds, coloured by country.....	107
Figure 4.7: Histogram showing distances between A) UK air sampling locations ($n = 800$) and B) all UK postcodes ($n = 1,771,918$) and the closest composting facility with open windrow or outdoor activity within 150 km.....	110
Figure 4.8: Plots showing the relationship between whether a sample grew <i>A. fumigatus</i> and A) sampling round that sample was collected in, B) maximum daily temperature at sampling location on sampling date, C) land cover classification of sampling location, D) percentage of arable land in the 2 km buffer surrounding sampling location, E) land cover classification of nearest composting facility with open windrow or outdoor activity (OW/OA), and F) proximity of sampling location to nearest OW/OA composting facility.	113
Figure 4.9: Geographically weighted regression coefficients for the relationship between increasing maximum daily temperature and likelihood of growing <i>A. fumigatus</i> at each sampling location for samples collected in A) summer, B) autumn, C) winter, and D) spring. All coefficients are shown on the left and coefficients for which the association was significant ($p \leq 0.05$) are shown on the right. Positive associations are shown in red and negative associations are shown in blue, with darker colours indicating stronger associations.....	116
Figure 4.10: Plots showing the relationship between the number of <i>A. fumigatus</i> colonies grown from samples and A) sampling round that sample was collected in, B) proximity of sampling location	

to nearest composting facility with open windrow or outdoor activity (OW/OA), and C) rural or urban location of nearest OW/OA composting facility to sampling location.	117
Figure 5.1: Histograms showing the distribution of numbers of A) <i>A. fumigatus</i> colonies and B) azole-resistant <i>A. fumigatus</i> colonies grown from positive samples.	136
Figure 5.2: Maps showing locations of soil samples collected by citizen scientists. Blue dots indicate samples that did not grow <i>A. fumigatus</i> colonies, green dots indicate samples that did and red dots indicate samples that grew azole-resistant <i>A. fumigatus</i>	138
Figure 5.3: Boxplot of maximum daily temperature at sampling locations on the date that soil sample was collected.	140
Figure 5.4: The 249 unique locations in the UK that soil samples were collected from, coloured by country.	141
Figure 5.5: Histogram showing distances between A) UK soil sampling locations ($n = 249$) and B) all UK postcodes ($n = 1,768,554$) and the closest composting facility with open windrow or outdoor activity within 90 km.	143
Figure 5.6: Plot showing the relationship between whether a sample grew <i>A. fumigatus</i> and the garden location from which the sample was collected. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.	144
Figure 5.7: Plot showing the relationship between the number of <i>A. fumigatus</i> colonies grown from a sample and the garden location from which the sample was collected. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.	146
Figure 5.8: Plot showing the relationship between whether a sample grew azole-resistant <i>A. fumigatus</i> and the garden location from which the sample was collected. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.	147
Figure 5.9: Plots showing the relationship between the number of azole-resistant <i>A. fumigatus</i> colonies grown from samples and A) land cover class of nearest composting facility with open windrow or outdoor activity (OW/OA) to sampling location, and B) rural or urban location of nearest OW/OA composting facility to sampling location.	148
Figure 6.1: Boxplot showing the maximum daily temperatures at the locations that air samples that grew azole-resistant <i>A. fumigatus</i> were collected from in each sampling round.	167

Acronyms and Abbreviations

ABPA	allergic bronchopulmonary aspergillosis
AIC	Akaike information criterion
AIDS	acquired immune deficiency syndrome
AMR	antimicrobial resistance
ANOVA	analysis of variance
AOD	acute oak decline
ARAF	azole-resistant <i>Aspergillus fumigatus</i>
<i>Bd</i>	<i>Batrachochytrium dendrobatidis</i>
BNG	British National Grid
<i>Bsal</i>	<i>Batrachochytrium salamandrivorans</i>
BWA	Burrows-Wheeler Aligner
CAPA	COVID-19 associated pulmonary aspergillosis
CF	cystic fibrosis
CFR	case-fatality rate
CFU/g	colony forming units per gram
CFU/m ³	colony forming units per cubic metre of air
CFU/ml	colony forming units per millilitre of liquid
CI	confidence interval
CLSI	Clinical & Laboratory Standards Institute
CNS	central nervous system
CO ₂	carbon dioxide
COPD	chronic obstructive pulmonary disease
COSHH	Control of Substances Harmful to Health
CPA	chronic pulmonary aspergillosis
CPP	crop pests and pathogens
Defra	Department for Environment, Food & Rural Affairs
diH ₂ O	deionized water
DMI	demethylation inhibitor
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
EA	Environment Agency
EtBr	ethidium bromide
EU	European Union

FERA	Food and Environment Research Agency
FVC	forced vital capacity
GATK	Genome Analysis Toolkit
GB	Great Britain
gDNA	genomic DNA
GDPR	General Data Protection Regulation
GIS	geographic information service
GLM	generalised linear model
Gt C	gigaton of carbon
GWR	geographical weighted regression
HIV	human immunodeficiency virus
IA	invasive aspergillosis
IAPA	influenza-associated pulmonary aspergillosis
ICL	Imperial College London
ICU	intensive care unit
ID	infectious disease
ISZ	isavuconazole
ITZ	itraconazole
LCM2019	2019 land cover maps
MAARA	Midlands Asthma & Allergy Research Association
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MEA	malt extract agar
MEGA	Molecular Evolutionary Genetics Analysis
MIC	minimum inhibitory concentration
MDR	multidrug resistance
MPS	mould preservation solution
mya	million years ago
NCBI	National Center for Biotechnology Information
NI	Northern Ireland
OED	Oxford English Dictionary
OPAL	Open Air Laboratories
OW/OA	open windrow or outdoor activity
PBS	phosphate buffered saline
PCP	pneumocystis pneumonia
PCR	polymerase chain reaction

PCZ	posaconazole
PDA	potato dextrose agar
PEG	polyethylene glycol
PHE	Public Health England
PPE	personal protective equipment
QC	quality control
RT	room temperature
RUBs	Really Useful Boxes
RMS	Royal Meteorological Society
SAFS	severe asthma with fungal sensitisation
SDA	Sabouraud dextrose agar
SDS	safety data sheet
SEPA	Scottish Environment Protection Agency
SNP	single nucleotide polymorphism
TB	tuberculosis
TBE	tris-borate-EDTA
TR	tandem repeat
UK	United Kingdom
UKCEH	UK Centre for Ecology & Hydrology
UKPN	UK Phenology Network
US	United States
UV	ultraviolet
VCZ	voriconazole
WGS	whole-genome sequencing
WNS	white-nose syndrome

Chapter 1: Fungi as Pathogens

Fungi belong to one of the largest, and most diverse, kingdoms of eukaryotic organisms on this planet. There have been ~120,000 species of fungi discovered but estimates of the total number in existence range from 2.2 to 3.8 million¹, and they are found in every temperate zone on Earth². In the tree of life, all cellular life forms are divided into 3 domains: Bacteria, Archaea and Eukaryota. Bacteria and Archaea are made up of prokaryotes – single-celled organisms that do not have a membrane-bound nucleus or organelles – whereas Eukaryota is made up of multicellular organisms whose DNA is contained within a nucleus and who possess organelles, such as mitochondria, that were once prokaryotes that formed symbiotic relationships with eukaryotes. The Kingdom of Fungi belongs to the domain Eukaryota along with the plant and animal kingdoms. An interesting study by Bar-On *et al.* (2018) showed how the ~550 gigatons of carbon (Gt C) in the world's biomass are distributed across these kingdoms: ~450 Gt C in plants, 12 Gt C in fungi and 2 Gt C in animals, of which 0.06 Gt C is in humans³. To put this in perspective, fungi have a combined biomass 200 times greater than the entire human race. Indeed the largest organism on Earth is *Armillaria ostoyae*, also known as “the humungous fungus”, which is a subterranean root pathogen spread across 2,400 hectares in northeast Oregon⁴. By analysing protein sequences of enzymes, scientists have estimated that fungi shared a common ancestor with animals that diverged from plants approximately 1000 million years ago (mya), and that animals and fungi diverged from each other ~965 mya⁵. Historically, before such analysis was possible, fungi were regarded as plants and studied by botanists⁶ and yet it turns out that fungi are more closely related to animals, and thereby humans, than they are to plants. The exciting implications of this are that chemicals produced by fungi to protect themselves could have pharmaceutical benefits for humans, however it also makes fungal infections difficult to treat because of the similarities between human and fungal cells.

While the focus of this thesis is a fungus that is an opportunist human pathogen, I felt it would be an injustice to this kingdom, and to the study of mycology, not to talk about some of the incredible things that fungi are capable of, as well as causing disease. Therefore, in Appendix 2 I have given an overview of the important roles that fungi play in medicine, food production, industry, bioremediation and in nature generally. In this chapter I will discuss the one-health

impact of fungi as plant, animal and human pathogens before focussing on *Aspergillus fumigatus* as the cause of lung disease in humans, which is the focus of my PhD.

1.1. Plant, animal and human pathogens

Pathogenic fungi constitute a truly one-health issue and currently shape our landscapes, threaten global food security and present a growing concern for public health. Global trade and international travel facilitate movement of pathogens between countries and continents, introducing them into previously naïve and susceptible populations. Our warming climate is expanding the range for current pathogens, and is adapting more fungal species for growth at mammalian body temperatures⁷. Improving medical knowledge is increasing the population of ageing and immunocompromised individuals who are most at risk from serious fungal diseases.

A holistic approach to combating fungal infections is required otherwise we may see the solution for one problem causing a problem elsewhere: as is being seen by an increase in lung infections caused by drug-resistant *Aspergillus fumigatus*, likely as a result of the environmental exposure of this fungus to fungicides being used to combat fungal plant pathogens. It is important that mycologists work together to share their findings and methods, because it is likely that progress made in one field can help to progress another.

1.1.1. Plant pathogens

We are faced with the challenge of feeding a predicted population of 9.7 billion by 2050⁸, yet currently 20% of crop yields are lost pre-harvest to viral, bacterial, fungal and oomycete pathogens and a further 10% post-harvest⁹. The predominant fungal and oomycete pathogens affecting calorie crops are *Magnaporthe oryzae* that causes rice blast, *Puccinia graminis* that causes wheat rust, *Ustilago maydis* that causes maize smut, *Phytophthora infestans* that causes potato late blight and *Phakospora pachyrizi* that causes soybean rust¹⁰. It is estimated that if severe outbreaks of these pathogens occurred simultaneously there would only be enough food to feed 39% of the world's population¹⁰. On the other hand, if average annual losses due to these pathogens could be mitigated there would be food for an additional 8.5% of the population¹⁰.

Fungal infections also threaten production of several cash crops grown in the tropics that cannot be grown elsewhere. Bananas are ranked as the world's important fruit and among the world's top 10 staple foods, with global exports reaching 18.1 million tons in 2017¹¹, but production is being devastated by Panama disease caused by *Fusarium oxysporum*¹². Cocoa beans have been enjoyed for >3,000 years, they provide income for over 40 million people and support a multibillion-dollar industry¹³, yet production is threatened by fungal pathogens *Crinipellis perniciosa* and *Moniliophthora roreri*¹⁴, with the former pathogen alone accounting for yield losses between 50 and 90%¹⁵.

The high water content and low pH of many fruits and vegetables gives fungi a competitive advantage over bacteria leading to spoilage of a wide range of crops¹⁶. *Botrytis cinerea*, also known as grey mould, can be pathogenic to over 200 species of plants including raspberries, strawberries, pears, peaches, cherries, plums, grapes, kiwi fruit, carrots, lettuce, peas and beans, while *Penicillium sp.* cause rot in citrus fruits, apples, pears and onions, and *Rhizopus stolonifera* and *Mucor piriformis* cause rot in raspberries, loganberries and strawberries¹⁶.

As well as direct damage to crops caused by fungal pathogens, crops can be ruined by the presence of mycotoxins, which are secondary metabolites produced by fungi in response to stress. There are approximately 400 mycotoxins known to be toxic when ingested, and 25% of global agricultural products have been reported as being contaminated with mycotoxins¹⁷. Mycotoxicosis can be acute or can have chronic effects such as reduced growth and development, immunosuppression and cancer, which predominantly occur in developing countries following continual low-level ingestion of mycotoxins¹⁸. Aflatoxins are produced by *Aspergillus sp.* and found in maize, peanuts, tree nuts and dried fruits, ochratoxin A is produced by *Aspergillus* and *Penicillium* species and found mainly in cereals, and *Fusarium*-produced fumonisins and trichothecens are found in maize and grain, respectively¹⁸. In addition to being a public health risk, mycotoxins result in economic losses due to ruined crops, lost trade revenues and livestock illness¹⁷.

Fungal pathogens are also shaping our landscapes by causing mass tree declines. *Ophiostoma ulmi* kills elm trees and is transmitted between trees by elm bark beetles: it has been destroying forest and urban elms across the US and Canada since its introduction in the 1930s, and the more virulent strain *Ophiostoma novo-ulmi* has decimated elms across all continents since its emergence in the 1960s¹⁹. *Hymenoscyphus fraxineus* is causing a major dieback in

common ash across Europe, which is expected to have serious economic and ecological consequences as ash produces high-quality timber, its leaf litter is easily decomposable and its foliage lets light through to understorey vegetation, thereby influencing the composition of forest floor plants²⁰. *Eucalyptus* trees are one of the most widely planted trees for commercial forestry worldwide due to their rapid growth and are planted across an estimated 20 million hectares in more than 100 countries; yet they are increasingly threatened by stem canker caused by *Botryosphaeriaceae*, *Ceratocystis*, *Chrysosporthe* and *Teratosphaeria* species²¹.

1.1.2. Animal pathogens

There are 625 species of fungi known to infect vertebrates²². In vertebrate wildlife, as in humans, fungi need to be able to grow at mammalian body temperature whilst avoiding destruction by both innate and acquired immunity, hence fungal infections are often associated with compromised immunity²². Aspergillosis, caused by *Aspergillus* species and most commonly by *A. fumigatus*, can affect a wide range of vertebrate and invertebrate hosts including fan corals, honeybees, reptiles, birds, cats, dogs, cows, horses and primates²³. It can take the form of allergic disease, localised infection or fatal disseminated infection most often stemming from the respiratory tract as a result of inhaling spores²³. Mammals can also suffer from mucormycosis, caused by fungi in the order *Mucorales*, when they encounter spores in their environment; either from inhaling the spores or ingesting them in large numbers²³. Candidiasis, caused by *Candida* species, affects birds, dogs, cats, ruminants, horses and pigs and can cause oral, gastrointestinal, pulmonary, cutaneous, skin, joint and ear infections²³. Other diseases that affect vertebrates include cryptococcosis, coccidioidomycosis, histoplasmosis, paracoccidioidomycosis and blastomycosis²³.

Of particular note are emerging fungal pathogens infecting wildlife that do not regulate their body temperature, such as amphibians and reptiles, or that hibernate, such as bats. *Batrachochytrium dendrobatidis* (*Bd*) causes chytridiomycosis and can infect over 700 species of amphibians; it has been found on every continent that amphibians occur, it is implicated in the decline of 43% of amphibian species and has caused the extinction of at least 100 species since 1980²⁴. The scale of these declines has led to chytridiomycosis being described as “the worst infectious disease ever recorded among vertebrates in terms of the number of species

impacted, and its propensity to drive them to extinction"²⁵. Amphibians use their skin to exchange respiratory gases, water and electrolytes and this osmoregulatory function is disrupted when *Bd* infects the skin layers, leading to electrolyte imbalance, cardiac arrest and death of the infected frog²⁶. A different emerging chytrid, *Batrachochytrium salamandrivorans* (*Bsal*), was identified as the cause of rapid population crashes in fire salamanders in the Netherlands such that by 2013 only 4% of the population remained²⁷. It is feared that its spread will rapidly result in the extirpation of susceptible salamander populations across Europe.

White-nose syndrome (WNS) is caused by the psychrophilic fungus *Geomyces destructans*²⁸ and gets its name from the white fungal growth on the muzzles, ears and wing membranes of hibernating bats²⁹. Since its discovery in a cave in north-eastern US in 2006, surveys suggest it is responsible for bat population declines of up to 75%²⁹. It is not yet known how WNS causes death but all infected bats have little or no fat reserves, which are essential for their survival during and after hibernation³⁰. Bats account for a fifth of mammalian species³¹ and are ecologically and economically important for pollination, seed dispersal and controlling insect populations³², many of which feed on agricultural crops or spread insect-borne diseases, making bat conservation a priority for species preservation, food security and public health.

There is indisputable evidence that many of the fungal diseases affecting wildlife and domesticated animals occur because of, or have been introduced by, human activity. For example, reptiles develop aspergillosis as a result of inappropriate temperature or humidity or poor hygiene within their enclosure, and cows can develop gastrointestinal mucormycosis following antibiotic treatment that disrupts their gut microbiota²³. *Bd* has been vectored around the world by the global pet trade, food industry and medical testing whereby species that are asymptomatic carriers of infection are released into wild populations or co-housed with susceptible species, and run-off from tanks containing fungal spores has entered the waterways³³. Furthermore, man-made deforestation and environmental pollution causes stress to wildlife making them more susceptible to disease¹⁰, therefore it is arguable that humans have an ethical obligation to save species that are in decline due to the fungal diseases we have introduced into their habitats.

1.1.3. Human pathogens

There are 200 fungal species capable of causing disease in humans²². These species infect billions of people worldwide and are responsible for 1.5 million deaths per year³⁴, which exceeds global mortality from breast cancer and malaria, and is comparable to tuberculosis (TB) and human immunodeficiency virus (HIV) death rates³⁵. Despite this, from 1997-2010 only 2% of the United Kingdom's (UK) £2.6 billion research budget for infectious disease was spent on medical mycology³⁶.

The most common fungal infections are skin mycoses caused by dermatophytes, estimated to affect 20-25% of the world's population³⁷. There are approximately 40 species of dermatophytes and they thrive in warm and humid conditions with temperatures of 25-28°C, such as human skin surface³⁷. There are regional differences in dermatophyte infections, with tinea pedis (athlete's foot) more prevalent in developed countries and tinea capitis (scalp infections) more common in poorer countries, although species are constantly moved around by tourism, migration and international sporting events³⁷.

The next most common fungal infections are oral and genital candidiasis, also known as thrush, caused by *Candida* yeast species. *Candida albicans* is responsible for the majority of cases of vulvovaginal candidiasis, which 70-75% of young women will experience at least once during their lifetime, 40-50% will experience more than once and 5-8% will suffer from four or more infections per year³⁸. *Candida* species are carried in the mouth of 30-45% of the healthy adult population and oral candidiasis is most likely to develop in infants (5-7%), cancer patients (20%) and acquired immune deficiency syndrome (AIDS) patients (9-31%)³⁹. Risk factors for oral candidiasis include age, diet, smoking, wearing dental prostheses and immune disorders³⁹.

The incidence of invasive fungal infections is much lower than that of superficial infections (skin mycoses and thrush) but result in mortality rates greater than 50%, despite antifungal treatments being available³⁶. More than 90% of these deaths are due to the four main fungal diseases, in order of prevalence: cryptococcosis, candidiasis, pneumocystis and aspergillosis, with the remainder of deaths due to coccidioidomycosis, histoplasmosis, mucormycosis, penicilliosis, paracoccidioidomycosis and blastomycosis³⁶.

Cryptococcosis infections in humans are caused by two species: *Cryptococcus neoformans* and *Cryptococcus gattii*. Cryptococcal infection often starts in the respiratory tract as a result of inhaling infectious propagules and can disseminate to most body sites, including skin, prostate, eyes and bones⁴⁰. Cryptococcal meningitis occurs when these encapsulated yeasts disseminate into the central nervous system (CNS) and has an annual incidence of 1 million infections, occurring predominantly in individuals with HIV/AIDS⁴⁰. Interestingly, cryptococcal meningitis is the fourth most deadly infectious disease after TB, malaria and diarrheal diseases (excluding HIV/AIDS) yet research on it receives 100-fold less funding than TB or malaria research and 25-fold less than diarrheal diseases⁴¹. Cryptococcal meningitis is responsible for 20 times more deaths than the bacteria *Neisseria meningitidis*, yet receives less than a quarter of the research funding⁴¹.

Invasive candidiasis, or candidemia, occurs when *Candida* species enter the bloodstream: it is associated with mortality rates of up to 60% and is the most common cause of fungal septic shock in intensive care unit (ICU) patients⁴². The majority of invasive infections are caused by five *Candida* species: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*⁴³. *Candida auris* is emerging as a major pathogen since its discovery in Japan in 2009⁴⁴ and is of concern for public health due to multidrug resistance (MDR), persistent candidemia and high mortality rates⁴⁵. *C. auris* hospital-associated infections have been reported in the UK, United States (US), Spain, South Africa, Kenya, India, Pakistan, Venezuela, Colombia and Israel⁴⁵.

The first record of pneumocystis was as a cause of pneumonia in premature babies and malnourished infants in orphanages in Europe after World War II and its prevalence increased during the HIV epidemic in the 1980s⁴⁶. Originally identified as a protozoan due to its lifecycle, it was reclassified as a fungus in 1988⁴⁷. Pneumocystis pneumonia (PCP) predominantly occurs in individuals who are immunocompromised as a result of HIV infection, immunosuppressant medication or chemotherapy and symptoms include shortness of breath, cough and low-grade fever, which can progress to hypoxia and respiratory failure⁴⁸. Treatment options are limited and pneumocystis is associated with 10-20% mortality rate for AIDS patients and 30-60% mortality rate in patients without AIDS⁴⁸.

Aspergillosis is caused by the environmental mould *Aspergillus fumigatus* and will be covered in greater detail in the following section, as it is the focus of this thesis.

1.2. *Aspergillus fumigatus* and aspergillosis

A. fumigatus is a saprotrophic fungus involved in degradation of decaying organic matter and plays an important role in recycling environmental carbon and nitrogen⁴⁹; it is estimated that life on earth would cease after a few decades without saprotrophic fungi because carbon and nutrients would become unavailable to the systems requiring them⁵⁰. Soil is the natural ecological niche of *A. fumigatus*⁵¹, the species is globally distributed⁵² and it is one of the most ubiquitous fungi in the world⁵¹. The fungus is thermotolerant and has an optimal growth temperature of 37°C but can grow at temperatures ranging from 12°C to 65°C and in pH ranging from 2.1 to 8.8⁵³. For many years *A. fumigatus* was believed to be an obligate asexual species⁵⁴ until O’Gorman *et al.* (2009) demonstrated a sexual reproductive cycle between isolates of complementary mating types⁵⁵. In the environment, reproduction of *A. fumigatus* is predominantly asexual: under favourable conditions a single uninucleate haploid spore divides mitotically to form up to 10⁹ new asexual spores within three to four days⁵⁶.

1.2.1. Dispersal of *A. fumigatus* in the air

A. fumigatus sporulates abundantly and each conidial head releases thousands of spores into its environment⁵¹. These spores are 2-3 µm in diameter and can be dispersed by the slightest air current due to their small size and hydrophobicity⁵³. Spores are found airborne in both outdoor and indoor environments⁵¹ and are protected from ultraviolet radiation by the melanin in their cell walls⁵³. They can also withstand temperatures of up to 70°C, can survive in liquid nitrogen for up to 18 years and can also tolerate prolonged dehydration; 25 years in anhydrous silica gel and 60 years when lyophilized⁵³.

Traditional methods for monitoring airborne *A. fumigatus* are using settle plates or an air sampler to suction pump air onto a sterile agar plate followed by incubation at 37°C or 42°C, colony counting and quantification as colony forming units per cubic metre of air (CFU/m³)⁵⁷. Many studies have monitored airborne *A. fumigatus* surrounding industrial composting sites^{58–63}, yet there have been few year-round studies looking at background levels of airborne *A. fumigatus* conidia. The Environment Agency stipulates that airborne *A. fumigatus* levels within 250 m of composting facilities must be no greater than 500 CFU/m³ above background levels⁶⁴, which are estimated to range from 3 to 20 CFU/m³ in the UK^{65,66}.

1.2.2. *A. fumigatus* in the soil

A. fumigatus is commonly found in composting vegetative material, compost from household waste, self-heating woodchip piles, sludge, sewage and mouldy hay⁶⁷. It plays an important role in the composting process because of its ability to degrade sugars, fatty acids, proteins, cellulose, pectin and xylene alongside its ability to proliferate during the initial thermogenic phase of compost when temperatures reach 40-60°C⁶⁸. Large-scale composting is becoming more common worldwide as the process diverts sewage sludge, agricultural waste and organic matter away from landfill and incineration⁶⁹. The role *A. fumigatus* plays in composting gives it ecological and economic importance, as compost is a useful product of waste management and compensates for the loss of humus in agricultural soils⁶⁸.

Soil-associated fungi such as *A. fumigatus* are also beneficial for agricultural yield as they restore soil fertility and organic matter content in degraded soils *via* nitrogen fixation and increased bioavailability of nutrients⁷⁰. As an example; phosphorus is the second most important nutrient for plant growth, after nitrogen. *A. fumigatus* has the ability to convert organic forms of phosphorus, such as phytin, phospholipids and nucleic acids, into inorganic forms that can be absorbed by plants using phosphatases⁷¹. Tarafdar *et al.* (1995) found that soil inoculation with *A. fumigatus* was associated with a positive and significant correlation increase in plant biomass⁷². *A. fumigatus* is able to grow rapidly on minimal growth media; only requiring a carbon source, a nitrogen source and trace elements⁴⁹.

1.2.3. *A. fumigatus* as a human pathogen

Studies have shown that humans inhale several hundred *A. fumigatus* conidia every day, and their small size allows them to bypass mucociliary clearance and reach the lung alveoli⁷³. In the majority of healthy individuals these spores are cleared by the innate immune system⁷⁴ but for some inhalation can trigger an immunoinflammatory response resulting in severe asthma with fungal sensitization (SAFS) or allergic bronchopulmonary aspergillosis (ABPA)⁷⁵. *A. fumigatus* is the predominant fungus cultured from airways of patients with cystic fibrosis (CF) and ~10% of CF patients suffer from ABPA, which is associated with airway damage and decline of lung function⁷⁶. If the spores are not cleared they can establish and grow in a pre-existing cavity in the lung, often caused by prior TB infection, bronchiectasis or pulmonary

infection, resulting in chronic pulmonary aspergillosis (CPA)⁷⁷. Other risk factors for CPA include prior diagnosis of ABPA, lung cancer, chronic obstructive pulmonary disease (COPD), previous thoracic surgery, diabetes, rheumatoid arthritis and alcoholism; only 5% of cases occur in individuals without underlying pulmonary or systemic disorders⁷⁸. In immunocompromised individuals, such as those with HIV/AIDS, undergoing chemotherapy or receiving organ transplant therapy, the immune system may fail to prevent spores from entering the bloodstream via the lungs resulting in a life-threatening disease called invasive aspergillosis (IA)⁷⁹. IA can also develop in apparently immunocompetent individuals and has been reported in patients with influenza and influenza pneumonia infections⁸⁰, which often results in delayed diagnosis and higher mortality because *A. fumigatus* colonisation is not expected⁸¹. COVID-19 associated pulmonary aspergillosis (CAPA) is occurring with alarming frequency, with retrospective case studies from Belgium, France, Germany and the Netherlands reporting CAPA in 20-35% of mechanically ventilated patients⁸². Mortality in 33 ventilated ICU patients with confirmed CAPA was 63.6% compared to 37.0% in 1,658 ventilated ICU patients without CAPA⁸².

It is difficult to estimate the burden of aspergillosis in the UK due to limitations in diagnosis, lack of national surveillance and few published studies⁸³. Pegorie *et al.* (2016) estimated the number of cases based on the UK's 2011 population of 63.2 million as follows: 178,000 (50,000-250,000) cases of ABPA in people with asthma, 3,600 cases of CPA, 2,900 cases of IA in non-critical care patients and 387-1,345 cases in critical care patients⁸³. These numbers are likely to increase in the UK as a result of the COVID-19 pandemic due to patients being treated with corticosteroids, which is a risk factor for influenza-associated pulmonary aspergillosis (IAPA) and is preliminarily associated with CAPA since 48% of confirmed CAPA cases had received corticosteroids⁸². Between 11th March and 14th July 2020, the UK National Mycology Reference Laboratory received samples from 719 critically ill COVID-19 patients in the UK and diagnosed probable/proven CAPA in 5% and possible CAPA in 15%⁸⁴. Of the 46 *A. fumigatus* isolates cultured from COVID-19 patients, three isolates were azole-resistant⁸⁴.

1.3. Drug-resistant *A. fumigatus*

1.3.1. During infection

Until the late 1980s, amphotericin B and caspofungin were the only drugs available to treat aspergillosis; they were given intravenously, had limited efficacy for ABPA or CPA and had a number of serious toxicities⁸⁵. The search for novel and less toxic antifungals led to the discovery of azoles, which are divided into imidazoles and triazoles that have two and three nitrogen atoms in their azole ring, respectively⁸⁶. Triazoles were favoured over imidazoles because of their improved toxicity profile, due to their greater affinity for fungal over human P450 enzymes at therapeutic concentrations⁸⁶. Henceforth, all mention of azoles will refer to triazoles and not imidazoles.

From 1990, first-generation azoles itraconazole (ITZ) and fluconazole became available, which can be given orally⁸⁷, and were followed a decade later by second-generation azoles voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ)⁸⁸. Now azoles are among the most widely used antifungal drugs due to their efficacy and low toxicity⁸⁹. A timeline of the approval of azole medical drugs is shown in Figure 1.1.

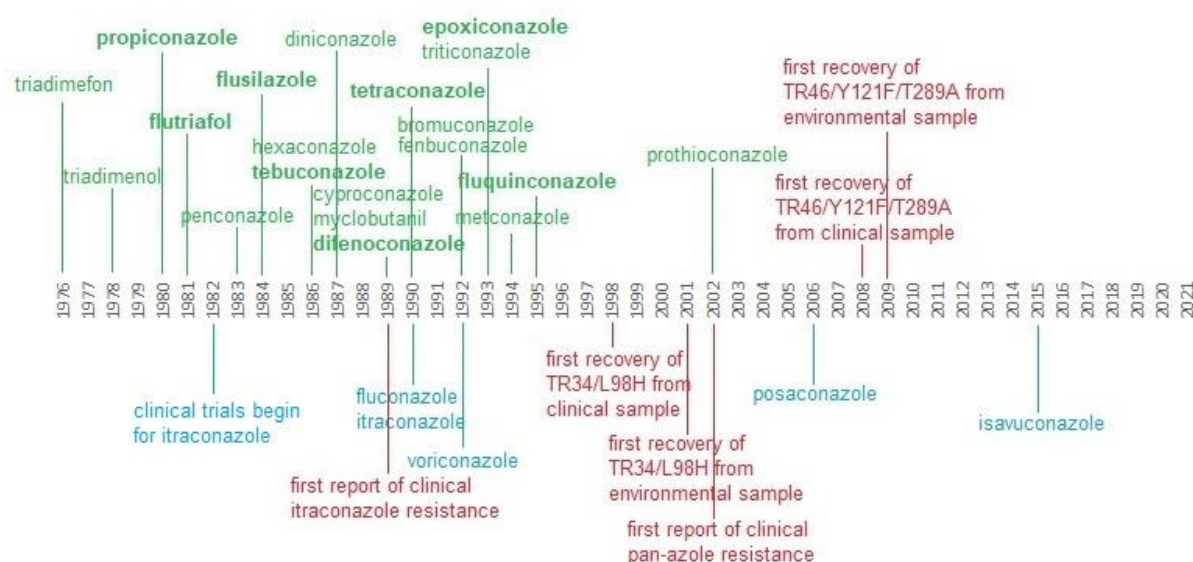


Figure 1.1 Timeline of dates for the global introduction of azole compounds into agriculture (shown in green) and medicine (shown in blue). The most-used agricultural azoles globally in 2008 are highlighted in bold and first reports of azole resistance are shown in red.

Drug resistance occurs when the minimum inhibitory concentration (MIC) - the lowest concentration of the drug that inhibits the growth of an organism⁹⁰ - exceeds the maximum concentration of drug that can be safely administered to the patient, such that the drug is unable to cure the infection⁹¹. The first clinical ITZ-resistant *A. fumigatus* isolates were cultured in 1989 from two patients in California who had been treated with ITZ⁸⁷ and ITZ-resistant *A. fumigatus* isolates were subsequently detected in Sweden in 1995⁹², in France in 1997⁹³, in the Netherlands in 1998⁹⁴, in the UK in 1999⁹⁵ and in China in 2002⁹⁶. From 2000, reports were published of clinical ITZ-resistance isolates with cross-resistance to VCZ and PCZ^{97–99} and multi azole-resistant *A. fumigatus* (ARAF) has now emerged as a therapeutic challenge in six continents¹⁰⁰. In the UK, the first ITZ-resistant clinical isolates were reported in 1999, ITZ- and PCZ-resistant isolates in 2000 and multi-azole resistant isolates in 2004¹⁰¹, as shown in Figure 1.2. By 2009, 20% of clinical isolates had resistance to at least one azole drug¹⁰¹. Azole-resistant infections are associated with treatment failure and increased case-fatality rates (CFR): CFRs up to 88% have been reported for azole-resistant IA¹⁰² compared to baseline mortality of 28.5% for azole-susceptible IA¹⁰³.

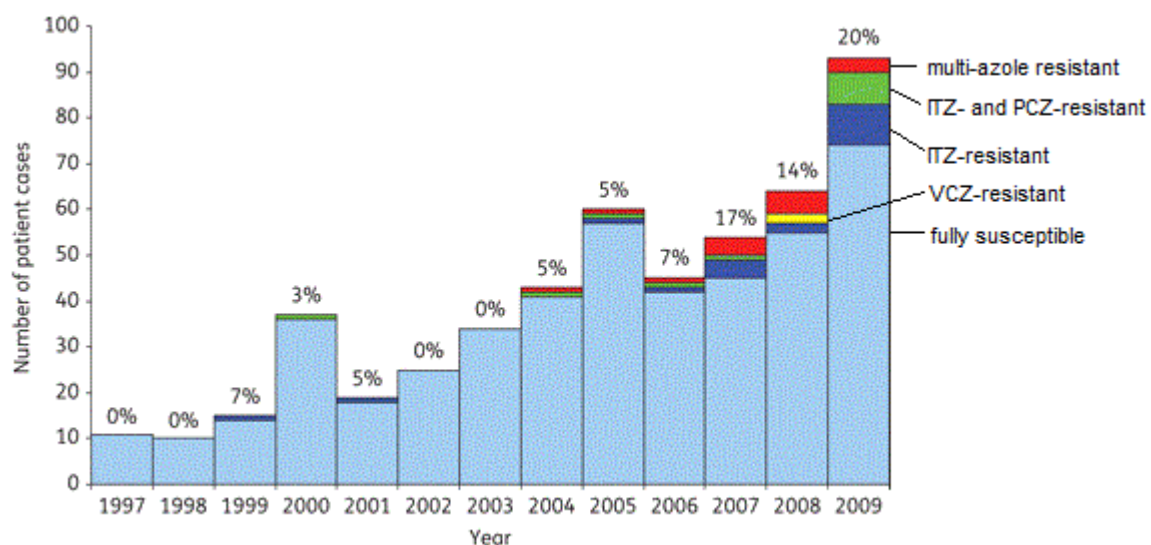


Figure 1.2 Barplot showing the number of patient cases with *A. fumigatus* infection in the UK between 1997 and 2009 and the percentage of patients with an azole-resistant infection, adapted from Bueid *et al.* (2010)¹⁰¹. ITZ=itraconazole, PCZ=posaconazole, VCZ=voriconazole.

1.3.2. In the environment

A. fumigatus is a saprotroph and is not phytopathogenic, but it is exposed to agricultural fungicides when they are deployed against plant pathogens such as *Fusarium*,

Mycosphaerella and *Aspergillus flavus*¹⁰⁰. The largest class of fungicides used in agriculture and horticulture are sterol demethylation inhibitor (DMI) fungicides; in 2005, this class made up 26.1% of total fungicide sales globally, and most of these fungicides were azoles¹⁰⁴. Azoles were first introduced into agricultural use in 1976 when triadimefon was approved and new azole fungicide formulations were subsequently released from 1978 through to 2002¹⁰⁴. As of 2008, the rankings of global azole sales were: tebuconazole, epoxiconazole, propiconazole, difenoconazole, flusilazole, tetraconazole, fluquinconazole, flutriafol¹⁰⁴. In the same year, the most sprayed azoles in the UK were: prothioconazole, epoxiconazole, tebuconazole, flusilazole, cyproconazole and propiconazole¹⁰⁵. Azoles are the popular choice for fungicides because of their broad-spectrum activity, slow development of resistance and high efficacy¹⁰⁴. A timeline of the introduction of azole fungicides into agriculture is shown in Figure 1.1.

Azole fungicides are applied as seed treatments, pre-harvest to prevent disease in grain crops and in grass-growing areas, such as golf courses, and are also applied post-harvest to delay spoilage in crops of carrots, cucumbers, strawberries and peppermint tea¹⁰⁶. In the UK, in 2008, the greatest use of azole fungicides was in wheat production, with 1.8 million hectares treated with epoxiconazole, prothioconazole and tebuconazole¹⁰⁵. In 2012, an assessment was carried out on the economic impact of the withdrawal of azole fungicides from wheat production in Europe, which estimated that within one year there would be loss of production of 9.9 million tons and 18.6 million tons by 2020¹⁰⁷. This equated to a loss of 2.4 billion euros in 2013 and 4.6 billion euros in 2020, and meant the European Union (EU) would no longer be self-sufficient for wheat production¹⁰⁷. Tebuconazole and propiconazole are used in wood preservatives to protect fencing, cladding, roofing and decking from wood decaying fungi, and propiconazole is used in adhesives, paints, leather, paper and textiles to prevent fungal growth¹⁰⁵.

1.3.3. Mechanisms of azole-resistance

The mode of action of azole drugs is to inhibit synthesis of ergosterol, the structural component of the fungal cell membrane in most yeasts and moulds¹⁰⁶. Polymorphisms in several genes involved in ergosterol biosynthesis, including *hmg1*, *erg6* and *cyp51B*, play a role in azole resistance but polymorphisms in *cyp51A* are frequently described as the main resistance mechanism¹⁰⁸. Azoles bind to lanosterol-14a-demethylase, an enzyme encoded by

the *cyp51A* gene that synthesises ergosterol from lanosterol by removing a methyl group¹⁰⁰. Polymorphisms in *cyp51A* lead to amino acid substitutions in the encoded protein and can result in structural changes to the enzyme, which in turn inhibits binding of azole drugs and results in drug-resistant isolates⁹⁵. Resistance is conferred by point mutations within the gene and can occur in combination with tandem repeats (TR) in the gene promoter region, which result in overexpression of *cyp51A*¹⁰⁹. A timeline of the discovery of common azole-resistance polymorphisms in *cyp51A* is shown in Figure 1.1.

Polymorphisms conferring resistance can occur in patients as a result of azole treatment and a number of *cyp51A* amino acid substitutions result in ITZ-resistance and varying MICs to VCZ and PCZ: F46, G54, G138, H147, M172, P216, M220, N248, D255, E427, Y431, G434 and G448, denoted by the amino acid substituted and the location of its codon⁹⁵. The most common multi azole-resistance mechanism reported is a 34 base pair TR in combination with a leucine-to-histidine substitution at codon 98, denoted as TR₃₄/L98H¹⁰⁸, which exhibits up to an eightfold increase in *cyp51A* expression¹¹⁰ and was first recovered from a clinical sample in the Netherlands in 1998⁹⁴. A different mutation TR₄₆/Y121F/T289A was subsequently discovered in clinical samples in the US in 2008¹¹¹ and in the Netherlands in 2009¹¹², defined by VCZ-resistance and a range of MICs for ITZ and PCZ. Further mutations reported from clinical samples include TR₅₃¹¹³, which was not accompanied by any *cyp51A* mutations, and TR₁₂₀¹¹⁴.

1.3.4. Evidence for environmental route of azole-resistance development

Initially, ARAf isolates were cultured from clinical samples from patients receiving long-term azole therapy until Verweij *et al.* (2007) recovered ARAf from patients who had no prior treatment with azoles¹¹⁵. A subsequent study that screened 2,062 *A. fumigatus* isolates collected from 1,385 patients in the Netherlands between 2007 and 2009 found that 64% of patients carrying ARAf had not received azole treatment in the previous three months¹⁰². As patient-to-patient transmission of *A. fumigatus* is rare¹¹⁶, the main route of infection is breathing airborne *A. fumigatus* spores. It is likely that subsequent azole treatment then selects for isolates that were already azole-resistant when inhaled¹⁰⁵.

TR₃₄/L98H was first recovered from an environmental water sample in Norway in 2001^{94,117} and TR₄₆/Y121F/T289A was first recovered from an environmental air sample in the Netherlands in 2009¹¹². Subsequent environmental sampling for *A. fumigatus* and MIC testing has discovered ARAf in Denmark¹¹⁸, France¹¹⁹, Germany¹²⁰, India¹²¹, Italy¹²², Kenya¹²³, Switzerland¹²⁴, Tanzania¹²⁵, the Netherlands¹²⁶ and the UK⁸⁹ with all studies that sequenced *cyp51A* detecting TR₃₄/L98H and many also detecting TR₄₆/Y121F/T289A. The global distribution of these polymorphisms suggests development of azole-resistance in *A. fumigatus* as a result of exposure to agricultural azoles because of the scale of fungicide deployment - in terms of tons of fungicides sprayed, area covered and frequency of application throughout the year - which could never be achieved by aspergillosis patients on azole therapy contaminating their environment with ARAf. Furthermore, isolates that become resistant in patients following azole therapy tend to have a single mutation in *cyp51A*¹²⁷ whereas van der Linden *et al.* (2011) reported that 90.2% of 82 clinical ARAf isolates carried the TR₃₄/L98H mutation¹⁰².

Snelders *et al.* (2012) identified five azoles frequently used in agricultural fungicides that have very similar molecular structures to the medical azoles, which showed cross-resistance and were all licensed for use prior to the first culture of TR₃₄/L98H ARAf from a clinical sample¹²⁸. According to pesticide usage data collected by the Food and Environment Research Agency (FERA), the cross-reactive azoles epoxiconazole, tebuconazole, propiconazole, difenoconazole and bromuconazole were the second, third, sixth, ninth and seventeenth most sprayed azoles in agriculture in the UK in 2008, respectively¹⁰⁵.

1.3.5. The growing problem of azole-resistant *A. fumigatus*

The challenges associated with ARAf are two-fold: 1) there is an increasing population of ageing and immunocompromised individuals, and now individuals with COVID-related lung damage, who are at-risk of aspergillosis infection, and 2) the pressures of feeding a growing population calls for greater reliance on pesticides such that the discontinued use of fungicides containing azoles is unlikely; even in the face of mounting evidence that they cause cross-resistance with medical azoles. In turn, there are two approaches needed to tackle the growing problem of ARAf. Firstly, research needs to prove that the increasing incidence of azole-resistant aspergillosis infections is due to environmental exposure of *A. fumigatus* to

agricultural azoles; to convince governments and fungicide manufacturers that new fungicides need to be ring-fenced for medical purposes only. Secondly, the ways in which individuals are exposed to *A. fumigatus* and ARAf spores need to be fully understood so that at-risk individuals can be advised how to minimise their exposures.

1.4. Project aims

The objectives of my PhD were 1) to determine the background levels of ARAf spores in the UK, and 2) to determine the main routes by which people living in the UK are exposed to *A. fumigatus* and ARAf spores. Widespread recovery of ARAf spores from UK air and soils will provide further proof for environmental acquisition of azole-resistance in *A. fumigatus*, and understanding people's exposures to *A. fumigatus* and ARAf spores may allow these exposures to be reduced – either by government interventions or behavioural changes.

To achieve these objectives, I asked citizen scientists resident in the UK to send me air and soil samples collected from their homes and workplaces. I then cultured *A. fumigatus* spores from these samples and tested them for susceptibility to tebuconazole; the third most-sprayed azole in agricultural fungicides in the UK. I extracted DNA from the tebuconazole-resistant isolates and sequenced the *cyp51A* promoter and gene regions to look for the polymorphisms causing resistance, and I used the sampling dates and locations for the samples that grew these isolates to look for associations between azole-resistance and seasonality, land-use and proximity to nearest composting facility. Unexpectedly, many of the tebuconazole-resistant isolates lost their resistance during their time in refrigerated storage so I undertook further investigations to ascertain whether this change was phenotypic or genotypic. An overview of the chapters in this thesis is given in Table 1.1.

Previous studies undertaken in the UK have collected air samples^{60,61,65,129,130}, soil samples¹³¹ or both^{89,132–134}, and have focussed on areas surrounding industrial composters^{60,61,129,130} or have been limited in their geographical spread^{65,131–134}. Asking citizen scientists to collect air and soil samples for this project achieved a higher number of samples with greater geographical spread than previous studies, and resulted in a large and valuable collection of *A. fumigatus* and ARAf isolates for ongoing research.

Chapter	Content
1 – Fungi as Pathogens	An introduction to fungi as plant, animal and human pathogens before focussing on aspergillosis lung infections in humans and the growing problem of azole-resistant <i>A. fumigatus</i> .
2 – General Methods	Methods for safe-handling, culturing, cryopreserving, DNA extraction and drug susceptibility testing of <i>A. fumigatus</i> , Sanger sequencing of <i>cyp51A</i> promoter and gene region, and obtaining environmental variables for sampling dates and locations to be included in association analyses.
3 – Air- and Soil-Sampling Mycology Citizen Science Projects	An introduction to past and present citizen science projects in the UK, followed by details of how the air- and soil-sampling projects for this PhD were organised, the number and geographical spread of samples collected, the number of <i>A. fumigatus</i> isolates grown and the benefits of citizen science participation for mycological research.
4 – Azole-Resistant <i>Aspergillus fumigatus</i> Spores in UK Air	An introduction to air sampling methodologies and previous studies sampling for <i>A. fumigatus</i> in UK air, followed by the numbers of tebuconazole-resistant <i>A. fumigatus</i> grown from UK air samples, their cross-resistance to medical azoles, the <i>cyp51A</i> mutations they contain and association analyses between samples that grew resistant isolates and relevant environmental factors.
5 – Azole-Resistant <i>Aspergillus fumigatus</i> Spores in UK Soil	An introduction to UK composting and gardening practices and previous studies sampling for <i>A. fumigatus</i> in UK soil, followed by the numbers of tebuconazole-resistant <i>A. fumigatus</i> grown from UK soil samples, the <i>cyp51A</i> mutations they contain and association analyses between samples that grew resistant isolates and relevant environmental factors.
6 – Is Climate Change a Concern for Azole-Resistant <i>Aspergillus fumigatus</i> ?	An introduction to the impact of climate change on infectious diseases, with particular focus on human and plant fungal diseases, followed by details of <i>cyp51A</i> sequencing, whole-genome sequencing and an experiment undertaken to investigate the effect of temperature on azole-resistance in <i>A. fumigatus</i> isolates.
7 – General Discussion	A brief discussion of the key findings of this thesis followed by suggestions for future directions of research based on these findings.

Table 1.1: An overview of the chapters in this thesis and a brief description of their contents.

Many of the citizen scientists who took part in my projects suffer from aspergillosis, or have a friend or family member who does, and are members of The Aspergillosis Trust. The Aspergillosis Trust and National Aspergillosis Centre helped to advertise my citizen science projects and I had increasing correspondence with these organisations throughout my PhD, which made me aware of the lack of awareness of aspergillosis: in the general public, in the scientific community and in the medical profession. Early diagnosis and treatment of aspergillosis is associated with better patient outcomes, yet a survey conducted by The Aspergillosis Trust revealed that diagnosis took between 1 to 5 years for 60% of 128

respondents (personal comms with Sandra Hicks and Gillian Fairweather at The Aspergillosis Trust). When I surveyed my ~1,200 Twitter followers in April 2020, of which the majority are scientists, many are mycologists and some are medical doctors, only 55% of 854 respondents replied “yes” when asked if they’d heard of aspergillosis or knew what it was. As a result of this, I have tried throughout my PhD to raise awareness of aspergillosis by giving talks at schools, community events and conferences, writing blogs and communicating on social media about my PhD research.

Undertaking a PhD in mycology has also highlighted a general lack of understanding and interest in the field amongst the majority of the population, whose knowledge of the topic is limited to mushrooms and athlete’s foot infection. I have given a talk to several school groups titled “Fungi: The Good, The Bad and The Ugly”, which touches upon many of the topics discussed in this introductory chapter, to show young people that there is so much more to mycology than what is covered in the school curriculum. While I have no way of measuring the success of my talks, many listeners have been excited and enthused by the content and I hope that I may have sowed a seed of interest in science, or even better mycology, in a school child who had not previously considered this as a future direction of study.

Chapter 2: General Methods

2.1. Safe handling of *Aspergillus fumigatus* in the laboratory

Prior to work commencing, risk assessments were completed for both Imperial College London (ICL) St Mary's campus and UK Centre for Ecology & Hydrology (UKCEH) Wallingford laboratories. These included risk assessments for handling of *A. fumigatus*, autoclaving media and pouring agar plates, and Control of Substances Hazardous to Health (COSHH) datasheets for the use of Distel™ surface disinfectant (Tristel Solutions Ltd, Cambridge, UK) and streptomycin (MilliporeSigma, Germany) and penicillin (MilliporeSigma, Germany) antibiotics.

All work was carried out in a category 2 hood, which was disinfected before and after use with Distel™ to prevent contamination of samples or the surrounding environment. An agar plate was kept open in the hood at all times while work was taking place to monitor for contamination. Personal protective equipment (PPE), including a fully-buttoned lab coat and gloves, was worn at all times in the laboratory and thorough hand-washing took place before leaving each room.

Sabouraud dextrose agar (SDA) plates without *A. fumigatus* or fungal growth of any kind were disposed of in a hazard waste bag, while plates without *A. fumigatus* but with other fungal growth present were parafilmed before disposal. All waste plates were double-bagged and taped shut before they were autoclaved. Once *A. fumigatus* colonies had been individually preserved each plate was sealed with parafilm and stored in 21L Really Useful Boxes (RUBs) in a walk-in fridge. All plates were kept within RUBs in the incubator, whilst being transported between laboratories and in the fridge and were only removed from these boxes in a category 2 hood. All RUBs were labelled with hazardous substances stickers.

2.2. Preparation of Sabouraud dextrose agar plates for culturing *A. fumigatus*

32.5 g of SDA (MilliporeSigma, Germany) was weighed into a clean 500 ml Pyrex media bottle, ensuring the bottle had an intact pouring ring. The bottle was filled to the 500 ml marker with deionised water (diH₂O), the cap screwed on and bottle shaken vigorously until agar powder

had completely dissolved. The autoclave tray was filled to the required level with diH₂O and the temperature sensor was fully submerged in a bottle of diH₂O inside the autoclave. The cap of the media bottle was loosened, the bottle was labelled using autoclave tape and placed inside the autoclave. The media program was run, whereby the autoclave reaches and maintains 121°C for 15 minutes to ensure sterility of agar. When the autoclave had cooled sufficiently for the door to unlock, heat-proof gloves were used to move the bottle(s) to a gently shaking incubator heated to 55°C and left to cool. 16x90 mm petri dishes or 25 cm³ Nunc™ EasYFlask™ cell culture flasks (ThermoFisher Scientific, UK) were laid out without lids in a category 2 hood and bottles of media were moved to the hood once cooled to 55°C. The next step depended on the type of media required:

GROWTH (SDA only) – use media straight from the autoclave, once cooled.

ANTIBIOTIC (SDA + penicillin + streptomycin) - weigh out 100 mg of penicillin and 200 mg of streptomycin and dissolve each in 1 ml of diH₂O before filter sterilising into media bottle and swirling to mix.

DRUG CONTROL (SDA + dimethyl sulfoxide) - pipette 60 µl of dimethyl sulfoxide (DMSO) into media bottle and swirl to mix.

DRUG (SDA + tebuconazole) - pipette 60 µl of stock tebuconazole (50 mg/L dissolved in DMSO) into media bottle and swirl to mix.

ANTIBIOTIC DRUG (SDA + tebuconazole + penicillin + streptomycin) - weigh out 100 mg of penicillin and 200 mg of streptomycin and dissolve each in 1 ml of diH₂O before filter sterilising into media bottle and swirling to mix. Then pipette 60 µl of stock tebuconazole (50 mg/L dissolved in DMSO) into media bottle and swirl to mix.

Media was poured carefully into the petri dish until the base of the dish was covered and was approximately one-third filled. When filling Nunc™ flasks the media was poured carefully into the flask opening until the 10ml line was reached, then the flask was tilted on its side. Media was left to dry for at least an hour before lids were replaced on dishes and flasks, which were stacked, re-wrapped in original packaging and sealed with tape to ensure sterility. Stacks were inverted, labelled with the date and stored at 4°C. Figure 2.1 shows plates and Nunc™ flasks drying in a hood after being poured.

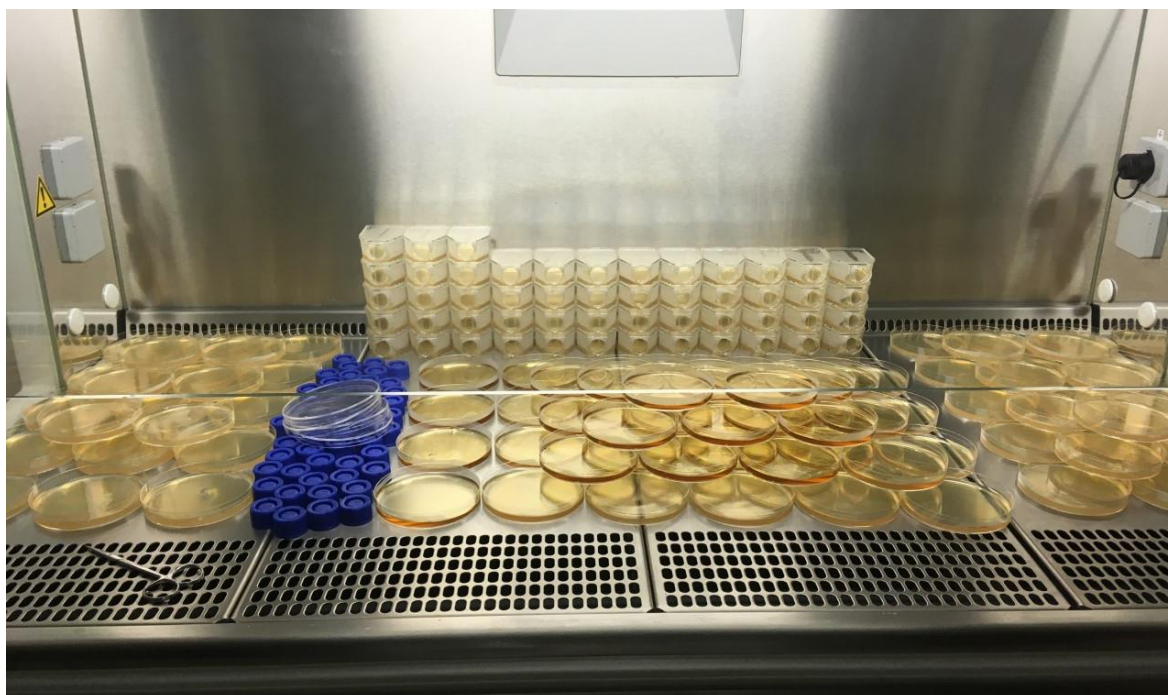


Figure 2.1: Plates and Nunc™ flasks left to dry in the hood for an hour once filled with Sabouraud dextrose agar.

2.3. Culturing *A. fumigatus* from air samples

The air samplers sent out to citizen scientists to collect *A. fumigatus* spores were MicroAmp™ clear adhesive films (ThermoFisher Scientific, UK) cut in half. Air samples were returned in Freepost envelopes along with a questionnaire completed by the citizen scientist, and were stored at room temperature until processing. In a category 2 hood, air samplers were cut to fit a 90 mm petri dish containing SDA plus penicillin (200 mg/L) and streptomycin (400 mg/L) to inhibit bacterial growth. The samplers were then placed sticky-side down on the agar and incubated at 43°C for 24 hours to select for growth of *A. fumigatus*, before the air sampler was removed and disposed of and the plate was incubated for a further 24 hours at 43°C. After 48 hours of incubation the plates were sorted and the number of *A. fumigatus* colonies recorded on a spreadsheet. Plates containing *A. fumigatus* were partially uncovered so the colonies could be picked one at a time using a sterilised wooden toothpick into a tube containing mould preservation solution (MPS; 0.2% agar and 0.05% Tween 20 in diH₂O), which was labelled with a code corresponding to the agar plate and stored at 4°C.

To ensure that air sampler plates could be linked to the relevant questionnaire containing meta-data, a permanent marker was used to write on the base of the plate the processing date, participant's initials and postcode, and a unique code containing project prefix

(SS18/AE18/WS18/SA19), participant number and location number. For example, the sample I collected from my home address for the first air sampling round was labelled 22/06/18, JS-OX14 5GE and SS18-001-01. The unique code was also written on the top of each questionnaire and data was input into an Excel spreadsheet.

2.4. Testing *A. fumigatus* isolates from air samples for tebuconazole-resistance

Isolates cultured from air samples were removed from the fridge and vortexed. In a category 2 hood, 5 µl of each isolate was pipetted into a petri dish containing SDA plus DMSO and another petri dish containing SDA plus tebuconazole (6 mg/L). A panel of previously whole-genome sequenced (WGS) *A. fumigatus* isolates were grown on SDA plates containing 0 mg/L, 4 mg/L, 6 mg/L, 8 mg/L and 16 mg/L of tebuconazole. 6 mg/L showed no growth of wildtype isolates, partial growth of TR₃₄/L98H isolates and full growth of TR₄₆/Y121F/T289A isolates, hence it was chosen as the screening concentration. To reduce plastic waste, petri dishes were divided into six segments and labelled on the underside with six isolate names, ensuring each isolate was pipetted into its corresponding segment. Plates were inverted, stacked in a RUB and incubated at 37°C for 48 hours. On the SDA plus DMSO plates, healthy *A. fumigatus* isolates grew to fill their segment of the plate producing a greeny blue colony 4-5 cm in diameter. If the isolate did not grow on SDA plus DMSO it was recorded as dead and if the colony grew more slowly or a different colour a record was made that it might not be *A. fumigatus*. If the isolate grew on SDA plus DMSO but not on SDA plus tebuconazole it was recorded as susceptible to tebuconazole, whereas if it grew on both plates it was recorded as tebuconazole-resistant and stored separately in the fridge for further testing. Once the resistance status of all isolates was recorded the SDA plus tebuconazole plates with growth were stored at 4°C and remaining plates were disposed of.

2.5. Culturing *A. fumigatus* from soil samples and testing for tebuconazole-resistance

Soil samples collected by citizen scientists were returned in a Freepost envelope with a completed questionnaire and were stored at room temperature until processing. 2 g of each sample was weighed out into a 15 ml Falcon tube and 8 ml of buffer (0.85% NaCl and 0.01% Tween 20 in diH₂O) was added. The tube was shaken vigorously to mix the soil and buffer and left to settle for 30 minutes to allow fungal spores in the soil to float to the surface. Initially, 200 µl from the surface of the buffer was pipetted onto an SDA plate containing penicillin (200 mg/L) and streptomycin (400 mg/L) and incubated at 43°C for 48 hours, then the number of *A. fumigatus* colonies was recorded and each colony was individually picked into a tube containing MPS. These isolates were refrigerated and tested for susceptibility to tebuconazole at a later date, as for isolates grown from air samples in section 2.4.

Due to the numbers of colonies grown from soil samples being far greater (up to 50 colonies) than the numbers grown from air samples (average 2-3 colonies), it was decided that this approach was unsustainable: both in terms of time and consumables required. From this point onwards, each sample was instead replica plated by pipetting 200 µl from the surface of the buffer onto an SDA plate containing penicillin (200 mg/L) and streptomycin (400 mg/L) and an SDA plate containing tebuconazole (6 mg/L), penicillin (200 mg/L) and streptomycin (400 mg/L). The plates were incubated at 43°C for 48 hours and the number of *A. fumigatus* colonies on both plates recorded. Only colonies growing on the tebuconazole plate were individually transferred into tubes containing MPS, labelled and stored at 4°C.

As for the air samples, each plate was labelled with the processing date, participant's initials and postcode and a unique code that was also written on the top of the accompanying questionnaire, to ensure soil samples could be matched to their collector. All unique codes for soil sampling began with SS19.

2.6. Cryopreserving *A. fumigatus* isolates

Tebuconazole-resistant isolates were removed from short-term storage in MPS at 4°C and vortexed. In a category 2 hood, 10 µl of each isolate was pipetted onto the centre of a 16x90 mm petri dish containing SDA only and the underside of the plate was labelled with the date

and isolate number. Plates were inverted, stacked inside a RUB and incubated at 37°C for 48 hours. In the hood, a sterilised wooden toothpick was used to remove a strip of mycelia and spores roughly 3 mm wide by 10 mm long spanning from the centre to the outside of the colony, which was carefully transferred to a 1.8 ml cryovial (STARLAB, UK) containing 1.5 ml of 50% glycerol solution (in diH₂O). Cryovials were then labelled with date and isolate name, shaken vigorously to disperse spores into solution, stored at 4°C overnight and transferred to -80°C for long-term storage.

2.7. Extracting DNA from *A. fumigatus* isolates

Tebuconazole-resistant isolates were removed from the fridge and vortexed. In a category 2 hood, 10 µl of each isolate was pipetted into a 25 cm³ Nunc™ flask containing SDA only, the lid was screwed on halfway to allow oxygenation of cultures and the flasks were labelled with the date and isolate name. The flasks were stacked inside a RUB and incubated at 37°C for 48 hours. In the hood, 2 ml of Tween solution (0.01% Tween 20 in diH₂O) was added to each flask, the lid was screwed on tightly and the flask shaken vigorously until the solution turned greeny blue with spores. 1 ml of this spore suspension was transferred to 1.5 ml tubes containing 0.03 g of 1 mm zirconia silica beads (Thistle Scientific Ltd, UK) and centrifuged at 8000 rpm for 5 minutes. In the hood, the supernatant was pipetted off and discarded and 60 µl of PrepMan™ Ultra (Applied Biosystems™, US) was added to each tube. Samples were homogenized for 45 seconds at 30 Hz using a BeadBeater, centrifuged at 14,500 rpm for 30 seconds, homogenized for 45 seconds and centrifuged for 30 seconds, before heating at 100°C for 10 minutes. Once removed from the heat-block, samples were left to cool for 2 minutes before being centrifuged at 14,500 rpm for 3 minutes. The supernatant was transferred to a 0.5ml DNA LoBind tube (Eppendorf, Germany), labelled with date and isolate name and stored at -20°C.

2.8. Testing *A. fumigatus* isolates for medical azole resistance

Isolates from air samples that were resistant to tebuconazole were taken to the Public Health England (PHE) Mycology Reference Laboratory in Bristol for antifungal drug susceptibility testing to the four medical azoles itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ) following Clinical & Laboratory Standards Institute (CLSI) guidelines.

The four azole drugs were stored in powder form at 4°C and were tested over the concentration ranges 0.06-32 mg/L. These steps detail the preparation and use of drug plates in accordance with the CLSI M38-A2 method:

2.8.1. Media preparation

20.8 g of RPMI medium (MilliporeSigma, Germany) was dissolved in 1,600 ml of distilled water. 69.06 g of MOPS buffer and 40 g of glucose were then added and stirred until dissolved. While stirring, the pH was adjusted to 7.0 using 1 mol/l sodium hydroxide and additional water was added to bring the final volume to 2,000 ml. Medium was filter sterilised and dispensed in 200 ml amounts into sterile containers, labelled with batch number and date and stored at 4°C. Two 10 ml aliquots of RPMI were taken from each sterile container and incubated at 35°C and at room temperature for two days. In the case of contamination, the batch of RPMI is discarded and re-made.

2.8.2. Preparation of antifungal drug dilutions

For ISZ and VCZ, the weight of powder to be dissolved in DMSO to create a 10,000 mg/L stock solution was calculated using their data sheets. 500 µl of this stock solution was added to 15.125 ml of RPMI medium to create a 320 mg/L stock solution.

Top label	Stock solution concentration (mg/L)	Volumes to be added to container (ml)		Drug concentration in container (mg/L)
		Stock solution	RPMI medium	
1	320	2	2	160
2	320	1	3	80
3	320	1	7	40
Use container 3 as stock solution for containers 4, 5 and 6.				
4	40	2	2	20
5	40	1	3	10
6	40	1	7	5
Use container 6 as stock solution for containers 7, 8 and 9.				
7	5	2	2	2.5
8	5	1	3	1.25
9	5	1	7	0.62
Use container 9 as stock solution for container 10, and discard 2 ml from container 9.				
10	0.62	2	2	0.31

Table 2.1: Details of dilutions to be carried out to ascertain required concentrations of drugs in containers 1 to 10, starting from a 320 mg/L stock solution in RPMI medium.

For ITZ and PCZ, 10 ml of polyethylene glycol (PEG) 400 was heated to 70°C and 16 mg of drug powder was dissolved in it to create a 1,600 mg/L stock solution. 1 ml of this stock solution was added to 4 ml of 50% PEG (2 ml of PEG 400 in 2 ml of sterile distilled water) to create a 320 mg/L stock solution.

Sterile containers were labelled from 1 to 10 and the 320 mg/L stock solution was diluted in RPMI medium as shown in Table 2.1.

At this point, the volume of solution in each container was 4 ml. 16 ml of RPMI medium was added to each container to give final concentrations ranging from 32 to 0.06 mg/L.

2.8.3. Preparation of microdilution plates

The 10 containers prepared in section 2.8.2 provided sufficient volume to fill 30 x 96-well microdilution plates. The plate lids were labelled with the drug name, batch number of RPMI medium, drug concentrations (0.06 – 32 mg/L) and expiry date (six months after preparation date). To enable the use of a multichannel pipette, 20 ml of drug-free RPMI medium was poured into a petri dish and 100 µl pipetted into columns 11 and 12 of each plate. The lowest drug concentration was pipetted into column 10 of each plate. This was repeated for the remaining drug solutions in order of increasing concentration such that column 10 contained 0.06 mg/L drug concentration through to 32 mg/L in column 1. Plates could then be stored at -20°C for up to six months.

2.8.4. Preparation of isolates for susceptibility testing

All isolates, along with the quality control (QC) *A. fumigatus* isolates NCPF 7097 and 7100, were sub-cultured onto potato dextrose agar (PDA) slopes 48 hours prior to testing. The spectrophotometer was switched on and left for 15 minutes, the wavelength set to 530 nm and “Mode” pressed to select % transmission. A number of sterile glass capped tubes equal to the number of isolates plus QC isolates were filled with 5 ml of 0.85% saline and caps were labelled with isolate and QC isolates names. A saline-filled tube was inserted into the spectrophotometer and “Calibrate” pressed to set to 100% transmission. A sterile inoculating loop was immersed in Tween 80, the top of a PDA slope carefully unscrewed and the loop gently touched to the colony to collect spores. The saline-filled tube was inoculated with the loop, vortexed for 15 seconds and reinserted into the spectrophotometer for a new

transmission reading, and this process was repeated until transmission was 80-82%. 0.1 ml of the spore suspension was transferred into 4.9 ml of RPMI medium and vortexed to create a final inoculum suspension concentration of 0.4 to 5×10^4 colony forming units per ml (CFU/ml).

2.8.5. Inoculation of microdilution plates

Plates were taken out of the freezer and left to thaw until the RPMI medium reached room temperature. The plate lids were labelled such that rows A and B of the first of each drug plate were for QC isolates 7097 and 7100 and subsequent rows were for other isolates. For each QC isolate and isolate, 100 μ l of the final inoculum suspension from section 2.8.4 was pipetted into columns 1 to 11 across the row labelled correspondingly. This process was repeated for each of the four drug plates and plates were incubated at 35°C for 48 hours. Figure 2.2A shows ITZ, PCZ, VCZ and ISZ drug plates filled with eight isolates for susceptibility testing and ready for incubation.

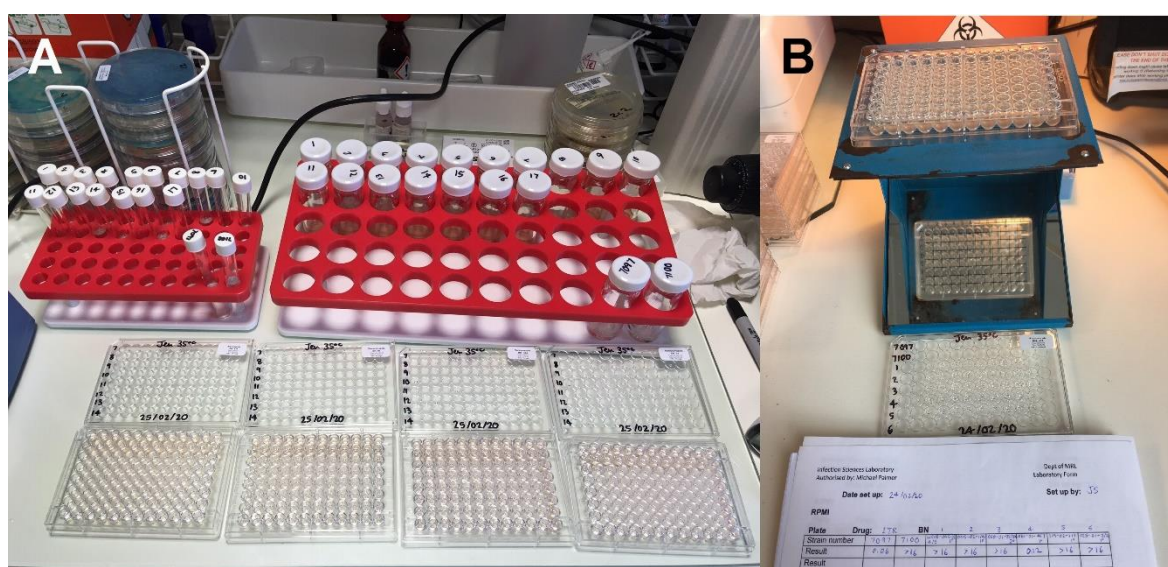


Figure 2.2: A) Itraconazole, posaconazole, voriconazole and isavuconazole drug plates filled with eight isolates ready for incubation, and B) a drug plate after incubation ready for isolate minimum inhibitory concentrations to be read and recorded.

2.8.6. Reading test results

Each plate was removed from the incubator and placed on the reading mirror, which was angled such that lamplight or daylight was illuminating the plate wells as shown in Figure 2.2B. There should not have been growth in column 12 as these wells were not inoculated with

isolates, so plates with growth in this column were discarded as contaminated. As column 11 was drug-free and inoculated this should have exhibited full growth of the isolate.

Before recording results for unknown isolates, it was ensured that the QC isolates produced results in the following range:

NCPF 7097:	ITZ	0.125 – 0.5 mg/L	NCPF 7100:	ITZ	>= 4 mg/L
	VCZ	0.125 – 0.5 mg/L		VCZ	0.25 – 1 mg/L

If the QC wells gave the expected results, MICs were then read for the remaining isolates. The MIC for *A. fumigatus* was defined as the lowest concentration at which there was 100% growth inhibition, so the well was completely clear of growth. MICs were recorded for each isolate against each drug on the record sheets provided.

2.9. Sanger sequencing of *cyp51A* gene and promoter region

2.9.1. Initial polymerase chain reaction

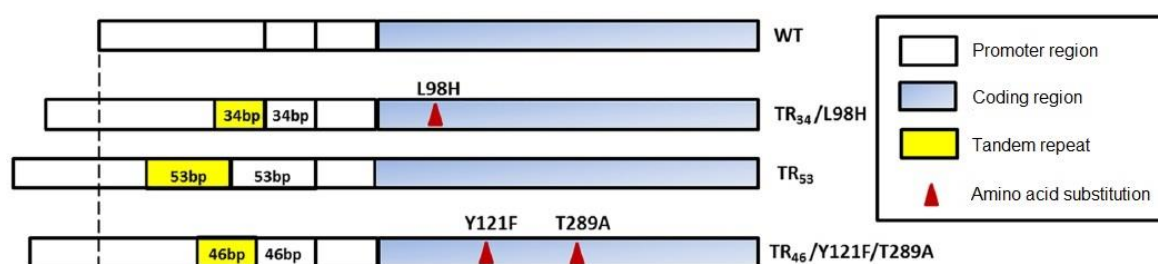


Figure 2.3: Illustration of *cyp51A* gene showing tandem repeats in the promoter region and amino acid substitutions caused by point mutations in the coding region, adapted from Zhang *et al.* (2017)¹³⁵.

Figure 2.3 is an illustration adapted from Zhang *et al* (2017)¹³⁵ showing the frequently-detected tandem repeats (TR) in the promoter region of *cyp51A* and accompanying amino acid substitutions caused by single nucleotide polymorphisms (SNPs) in the coding region. To amplify both the promoter and coding region of *cyp51A* two primer sets were used with different master mix solutions and polymerase chain reaction (PCR) conditions, detailed in Table 2.2. FIREPol® Master Mix (Solis BioDyne, Estonia) was used to amplify the promoter region because it is considerably cheaper than a high-fidelity Taq, which was not required to characterise TRs, and the all-in-one master mix format saved time and reduced pipetting error making the multiple runs more consistent. The primers for the promoter region were designed by a colleague and are not yet published (personal comms, Asmaa Alghamdi). Q5®

High Fidelity DNA Polymerase (New England BioLabs Ltd., UK) was used to amplify the coding region because the aim was to characterise SNPs resulting in amino acid substitutions in the translated protein, therefore accuracy was crucial. All primers were ordered from Invitrogen (Thermo Fisher Scientific, UK) at a standard concentration of 100 μ M in water and the DNA for each sample used in the PCRs was diluted tenfold in diH₂O from its extracted DNA. Deoxynucleoside triphosphate (dNTP; New England BioLabs Ltd., UK) were initially used at 10 μ M concentration but a subsequent order arrived at 40 μ M concentration, so 0.5 μ l was instead used in the per sample master mix solution for the coding region PCR and the volume of diH₂O increased to 19.5 μ l.

Primers (5' to 3')	Per sample master mix solution	PCR conditions
Promoter region: Forward- GGACTGGCTGATCAAACATATGC Reverse- GTTCTGTTCGGTTCCAAAGCC	10 μ l FIREPol® Master Mix 10 μ l forward primer (1.5 μ M) 10 μ l reverse primer (1.5 μ M) 18 μ l diH ₂ O 2 μ l DNA	95°C, 5 minutes 98°C, 20 seconds 65°C, 30 seconds 72°C, 30 seconds 72°C, 5 minutes Hold at 10°C }x30
Coding region: Forward- ATGGTGCCGATGCTATGG Reverse- CTGTCTCACTTGGATGTG	0.2 μ l Q5® DNA Polymerase 2 μ l dNTPs (10 μ M) 1 μ l forward primer (10 μ M) 1 μ l reverse primer (10 μ M) 33.8 μ l diH ₂ O 10 μ l Q5 buffer (5X) 2 μ l DNA	94°C, 2 minutes 94°C, 30 seconds 60°C, 45 seconds 72°C, 45 seconds 72°C, 5 minutes Hold at 10°C }x35

Table 2.2: Details of the primers, master mix solutions and PCR conditions used to amplify the promoter and coding regions of *cyp51A*.

While the PCRs were running, a 1.5% agarose gel was poured and left to dry. The gel was made by adding 3 g of agarose (Merck KGaA, Germany) to 200 ml of 1X tris-borate-EDTA (TBE) buffer in a glass conical flask, diluted from 10X TBE buffer (ThermoFisher Scientific, UK) in diH₂O, and heating in the microwave at full power, swirling regularly, until the powder had dissolved. 5 ml of ethidium bromide (EtBr; Merck KGaA, Germany) was then added, the flask swirled to disperse the EtBr and the gel solution poured into a gel tray containing two rows of 51-tooth combs. The gel tank was filled to the maximum fill line with 1X TBE buffer. When the PCRs finished, the combs were carefully removed from the gel and the gel was placed into the gel electrophoresis tank, making sure the black wire (cathode) was above the gel and the red wire (anode) was below so the DNA migrated towards the positively charged anode. 3 μ l

of each promoter region PCR product was loaded directly onto the gel as the FIREPol® Master Mix contains blue and yellow tracking dyes, whereas 3 µl of each coding region PCR product was first mixed with 0.6 µl of 6X gel loading dye (New England BioLabs Ltd., UK) before loading onto the gel. 3 µl of 1 kb plus DNA ladder (New England BioLabs Ltd., UK) was added to the first and last well of each row and the gel was run for 20 minutes at 120 volts. The gel was then imaged using a Bio-Rad GelDoc (Bio-Rad Laboratories, UK) in conjunction with Image Lab software (Bio-Rad Laboratories, UK).

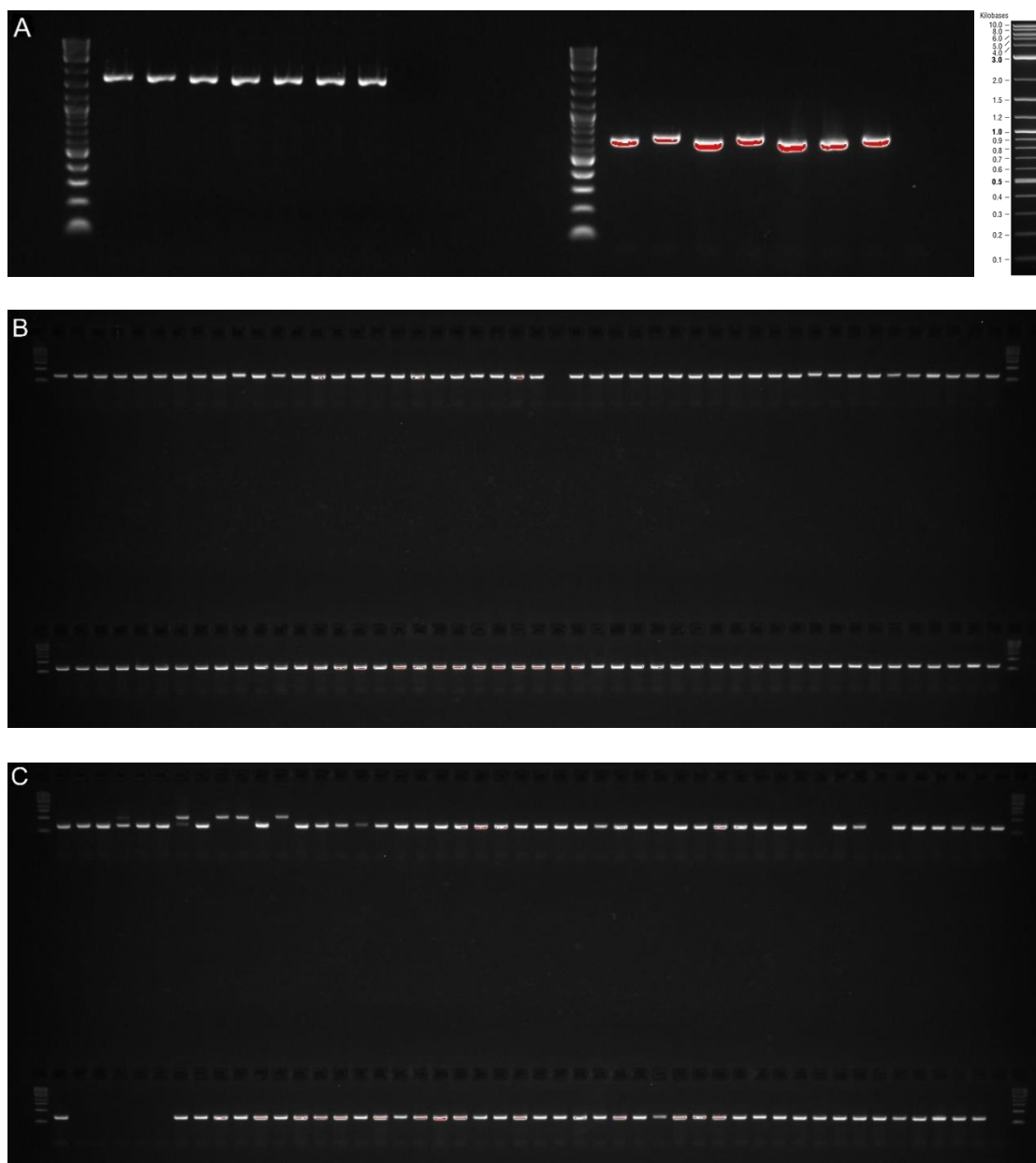


Figure 2.4: Electrophoresis gel images showing A) amplicons from *cyp51A* coding region PCR (left) and *cyp51A* promoter region PCR (right) for the same seven DNA samples, B) a 96-well plate of samples from promoter region PCR where all samples have amplified except for the negative control, and C) a 96-well plate of samples from promoter region PCR where several samples have not amplified.

2.9.2. Sanger sequencing

```

1   GTCGAGTGCAGGACTGGCTGATCAAACATGCTCATGCACTGTCTTCTCTACATAACAGAATACTGGGCAGCGGGCTGGA
    promoter region - forward primer →
83  GATACTATGGCTTTCATATGTTGCTCAGCGGCAGCATTCTGAAACACGTGCGTAGCAAGGGAGAAGGAAAGAAGCACTCT
163 GAATAATTTAACTGTTCTCTCTAGAAAAAACTCATGAGTGAATAATCGCAGCACCCTTCAGAGTTGTCTA34 bp tandem repeatGAATCACGC
245 GGTCCGGATGTGTGCTGAGCCGAATGAATCACGCGGTCCGGATGTGTGCTGAGCCGAATGAAAGTTGCCTAATTACTAAG
325 GTGTAGTTCAGCATACCATAACCCCTAACTCATACTACGGTAGGTAGATCTACTTACCTATGAACCTATATTGGTAGGTAG
407 GTGAATATAAAATACAGCATGGAACATGTTTTTCATTAGCTGGTCTCTCATTGCTCCTTGCTCCTAGGCCCTTAAGGAATCCAG
489 TATATGAAATAATCCCTCTTATCCATTTTCTCTCTATTCTTTTTCATTCCCTCATCACTGCAACTCTAATCCTCGGGCTCACCC
574 TCCCTGTGTCTCTCGAAATGGTGCCGATGCTATGGCTTACGGCCTACATGGCCGTTGCGGTGCTGACGGCAATCTTGCTC
    coding region - forward primer →
655 AATGTTGTTTATCAATTATCTTTTGGCTTTGGAACCGAACAAGCGCAATGGTCTTTCATTGGGTCCCATTCTGGGTGA
    ← promoter region - reverse primer
737 GTACCATCAGTTACGGGATTGATCCCTACAAGTTCTTTGCGTGACAGAGAAAAGGCAAGTCTCAAGATTGTAGTTTGAC
818 ATTCATTCTGGGCGCATTGCTGAGTATTGCTTTCTAACCGGCAGTATGGCGATATCTTCACTTTTATACTGTTGGGTCAAA
901 AAACCACAGTCTACCTGGGCGTTTCAGGGGAACGAGTTTATTCTCAACGGCAAGCTL98H: T to ACAAGGATGTCAATGCGGAAGAGGTCT
981 TATAGTCCATTGACGACCCCCGTTTTTCGGATCGGACGTGGTGTATGATTGTCCCAATTCCAAGCTGATGGAGCAGAAAAAG
1062 TTCATCAAGTACGGCTTGACTCAGTCTGCGTTAGAGTCTCATGTGCCACTTATTGAGAAGGAGGTTTTGGACTATCTGCGC
1143 GATTACCGAACTTTCAAGGCTCGTCCGGCCGATGGACATCTCTGCGGCAATGGCTGAGATTACCATTTTACCCTGCTGCT
    coding region - sequencing primer →
1224 CGAGCCCTCCAAGGCCAGGAAGTTTCGTTCCAACTCACGGCTGAGTTCGCTGACCTCTATCATGACCTGGACAAGGGCTTT
1305 ACTCCCATCAATTTTATGCTACCGTGGGCCCCATTGCCGCATAACAAGAAGCGAGATGCTGCTCATGCGCGCATGAGGTCA
1386 ATCTACGTTGACATCATCAATCAGCGCCGTCTTGACGGTGACAAGGACTCTCAGAAATCAGACATGATATGGAACCTGATG
    ← coding region - sequencing primer
1467 AACTGCACATACAAAACGGCCAGCAAGTGCCTGATAAAGAGATTGCGCACATGATGATAACCCTGTTGATGGCTGGTCA
1547 GCATTGCTCTTCGTCCATCAGCGCCTGGATTATGCTGAGACTGGCCTCACAGCCAAAAGTCTCGAAGAGCTGTATCAGGA
1628 ACAGCTGGCCAATCTTGGCCCCGCCGGGCCAGACGGCAGTCTTCTCCGCTCCAGTACAAGGATCTTGACAAACTTCCCTT
1709 CCATCAACATGTTATTCGTGAAACCTTACGGATTCACTCTCTATTCACTCTATCATGCGCAAGGTGAAAAGCCCCCTTGCCC
1791 GTTCCCGGGACCCCTTACATGATTCTCCCGGTGCGGTGCTCCTTGCTTCACTGGAGTGACAGCCCTCAGCGACGAACACT
1873 TCCCCAATGCTGGGTGCTGGGATCCCCATCGCTGGGAGAACCAGGCTACTAAGGAGCAGGAGAACGACGAGGTTGTGCA
1952 CTACGGTTACGGCGCCGTCTCCAAGGGCACGTCAAGTCCCTATCTTCCGTTTGGTGTGCGCCGACACCGCTGTATCGGCGA
2033 GAAATTCGCTTATGTCAACCTTGGTGTGATTCTGGCGACCATTGTGCGCCACCTGCGACTTTTCAACGTGGATGGAAAGAA
2114 AGGAGTCCCTGAAACTGACTATTATCCCTCTTTTCGGGCCCATGAAGCCAAGCATCATCGGCTGGGAGAAGCGGTGCA
2194 AAAACACATCCAAGTGAGACAG
    ← coding region - reverse primer

```

Figure 2.5: DNA sequence of *cyp51A* promoter and coding regions, with primers used to amplify and sequence the promoter region highlighted in turquoise and primers used to amplify and sequence the coding region highlighted in yellow. The insert point for the 34 base pair tandem repeat (TR₃₄) and the single nucleotide polymorphism that results in the L98H amino acid substitution are shown for reference.

Once any samples in a 96-well plate that did not amplify were replaced with samples that did amplify, the samples were transferred to a skirted PCR plate (Merck KGaA, Germany), labelled with a plate barcode and sent to Genewiz® (UK) for Sanger sequencing. The plate was accompanied by 100 µl of a primer diluted to 10 µM in diH₂O. To sequence the promoter region the forward primer 5'- GGACTGGCTGATCAAACATATGC-3' was used. The coding region was sequenced in two parts using primers with binding sites located in the middle of the amplicon: the first 730 bp were sequenced using the primer 5'-CTGATTGATGATGTCAACGTA-3' and the second ~1000 bp were sequenced using an upstream primer 5'-GATTCACCGAACTTTCAAGGCTCG-3'. The DNA sequence of *cyp51A* is shown in Figure 2.5 with the primers used to amplify and sequence the promoter region and coding region highlighted in turquoise and yellow, respectively.

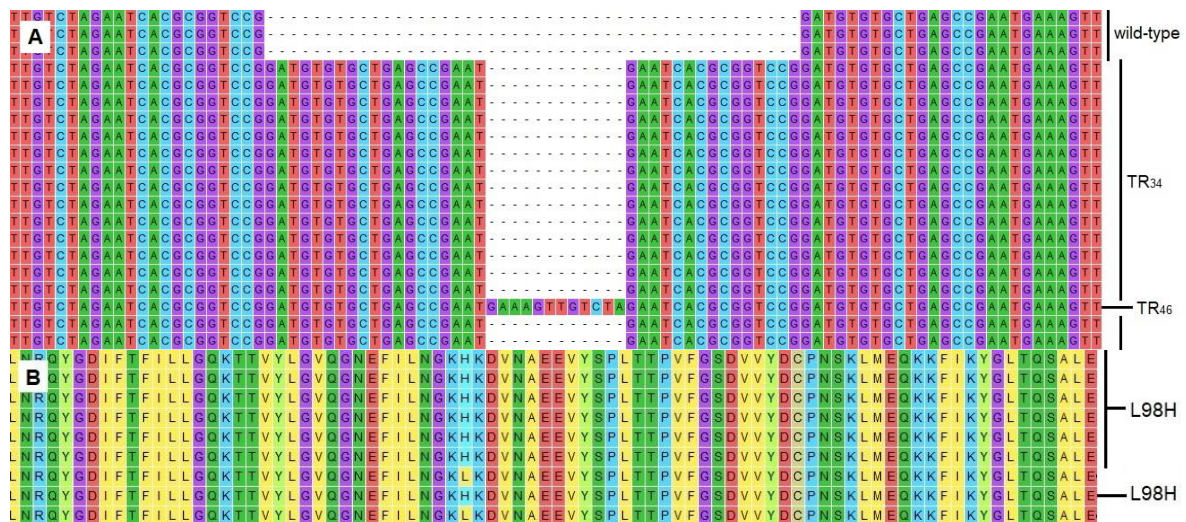


Figure 2.6: A) DNA alignment of the *cyp51A* promoter region for 20 samples with their wild-type, TR₃₄ or TR₄₆ status annotated on the right-hand side, and B) protein alignment for nine samples translated from a DNA alignment, with L98H amino acid substitutions annotated on the right-hand side.

When the plate had been sequenced the results were available for download from the Genewiz® website as a FASTA file, which was opened in Molecular Evolutionary Genetics Analysis (MEGA) software (Penn State University, US). Samples that had failed to sequence were removed and the remaining sequences were aligned using the Clustal multiple sequence alignment tool. The aligned DNA sequences were sufficient to detect TRs in the promoter region, whereas the aligned DNA sequences were then translated into a protein sequence for the coding region to determine whether any SNPs present resulted in amino acid substitutions. Figure 2.6A shows a DNA alignment of the promoter region for 20 samples; of

which three are wild-type, 16 contain TR₃₄ and one contains TR₄₆. Figure 2.6B shows a protein alignment for nine samples translated from a DNA alignment; of which seven samples contain the L98H amino acid substitution.

2.10. Confirming identity of isolates as *A. fumigatus*

2.10.1. Macroscopic appearance

Colonies cultured from air and soil samples were initially identified as *A. fumigatus* by their macroscopic appearance when grown on SDA plates, as shown in Figure 2.7A. This is typically a blue green colour and matt appearance of the colony from above, a lack of colouration of agar beneath or around the colony, ridges formed in the SDA on the underside of the colony and a spherical shape of the colony. The identity of these colonies as *A. fumigatus* was also inferred by their ability to grow at 43°C, which is unusual for the majority of environmental fungi.

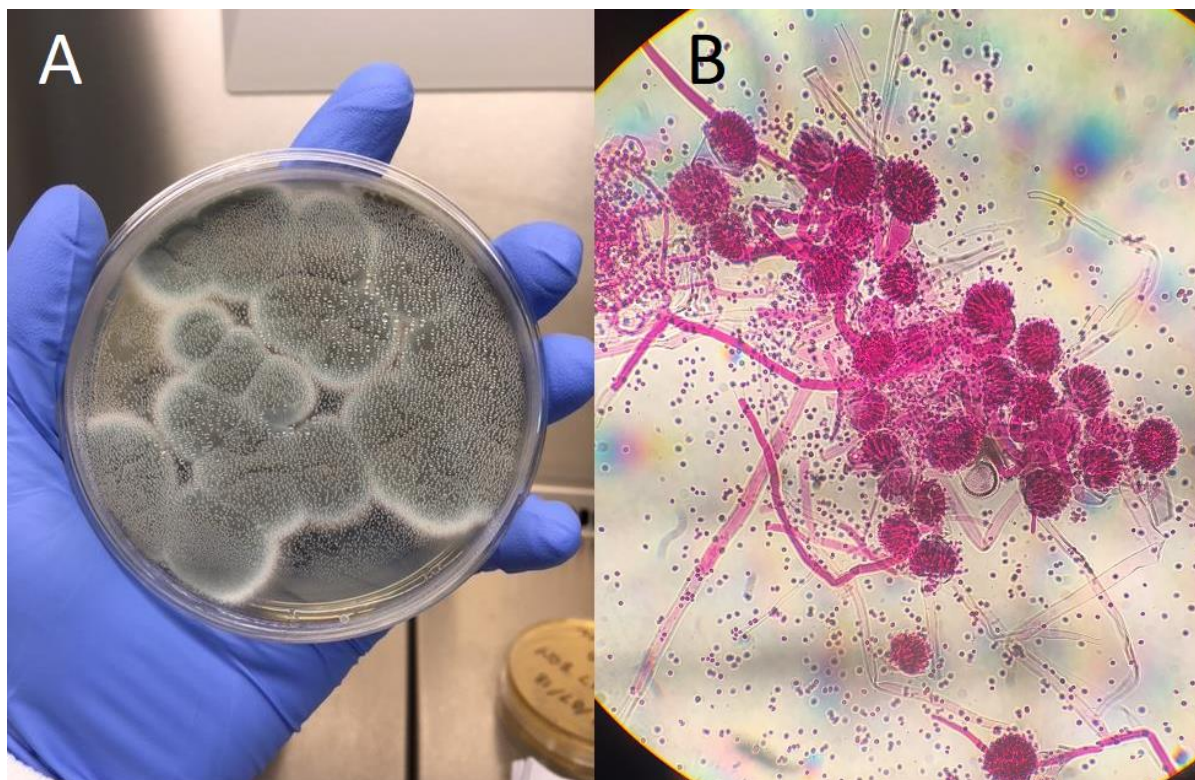


Figure 2.7: A) Macroscopic appearance of *A. fumigatus* as blue green colonies after 48 hours of growth on Sabouraud dextrose agar at 43°C, and B) microscopic appearance of *A. fumigatus* spore heads at 400X magnification.

2.10.2. Microscopic appearance

As mentioned in section 2.4, if an isolate didn't resemble *A. fumigatus* when it was cultured onto SDA control plate for susceptibility testing to tebuconazole it was marked on the spreadsheet for further investigation. A microscope slide of the colony was prepared and the structures of the fungal spore heads were examined at 100X and 400X magnification, using Campbell *et al* (2013)¹³⁶ to aid with identification. Figure 2.7B shows the appearance of *A. fumigatus* spore heads at 400X magnification. Using this approach, two isolates were reclassified as *A. nidulans*, which has a slightly greener macroscopic appearance than *A. fumigatus* but is otherwise similar until it is examined microscopically.

2.10.3. Matrix assisted laser desorption ionization-time of flight mass spectrometry

Several isolates no longer macroscopically resembled *A. fumigatus* when they were cultured on PDA for susceptibility testing at PHE (section 2.8.4) so they were identified using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Using this approach, 10 isolates were reclassified as *A. lentulus*, which is identical to *A. fumigatus* in both macroscopic and microscopic appearance. The approach also confirmed the identity of the two isolates reclassified by their microscopic appearance as *A. nidulans*.

2.10.4. Sanger sequencing of beta-tubulin gene

When the PCR amplicons of the 12 isolates reclassified as *A. nidulans* and *A. lentulus* were sent for Sanger sequencing they were either determined as wild-type or failed to sequence. As only tebuconazole-resistant isolates had DNA extracted for sequencing, subsequent samples that were determined as wild-type or failed to sequence underwent a further PCR using primers for a conserved beta-tubulin gene used to identify fungi: Bsens (5'-ATCAC(A/T)CACTCICTIGGTGGTGG-3') and Brev (5'-CATGAAGAA(A/G)TGIAGACGIGGG-3'). This PCR used the same master mix as the promoter region PCR (Table 2.2) and the following PCR conditions: 94°C for 3 minutes for initial denaturation, 30 cycles of 94°C for 15 seconds for denaturation, 55°C for 30 seconds for annealing, 68°C for 30 seconds for extension, and final extension at 68°C for 3 minutes before holding at 10°C. The forward primer Bsens at a

concentration of 10 μ M was sent to Genewiz® with the PCR amplicons to be used for Sanger sequencing. The sequences were then uploaded to the National Center for Biotechnology Information (NCBI) website and aligned to sequences in the database with species identification to find the sequence(s) with the highest identity match.

2.11. Whole-genome sequencing of *A. fumigatus* isolates

2.11.1. DNA extraction and whole-genome sequencing

Fresh DNA extractions were made for isolates being sent for whole genome sequencing (WGS) as high-quality DNA was required. Isolates for WGS were brought back from cryopreservation at -80°C by pipetting 20 μ l into 25 cm³ Nunc™ flasks containing SDA and incubating at 37°C for 48 hours. Spores were then harvested by washing the surface of the SDA with 10 ml of phosphate-buffered saline (PBS) plus 0.01% Tween 20, 1.8 ml of spore suspension was added to 2 ml FastPrep tubes (MP Biomedicals, US) and tubes were centrifuged at 5,000 rpm for 10 minutes. DNA extraction then commenced by discarding the supernatant and resuspending the pellet in 300 μ l of lysis solution and 1 μ l of proteinase K from the MasterPure™ Complete DNA and RNA Purification Kit (Lucigen, US). The kit protocol was followed, including an additional bead-beating step using a FastPrep-24™ instrument. Extracted genomic DNA (gDNA) was then purified using a DNeasy Blood and Tissue Kit (Qiagen, Germany) and the DNA concentration was measured using a Qubit fluorometer and Qubit dsDNA BR Assay kit (ThermoFisher Scientific, UK). A NanoDrop™ spectrophotometer (ThermoFisher Scientific, UK) was used to assess DNA purity by checking that the ratio of absorbances at 230/260 nm and 260/280 nm were 1.8-2.0. Purified gDNAs were stored at -20°C prior to being sent to Earlham Institute (UK) where gDNA libraries were constructed, normalised and indexed. Libraries were then run on a NovaSeq 6000 SP v1.5 flow cell to generate 150 bp paired-end reads.

2.11.2. Bioinformatic analysis

Raw paired-end reads were quality checked using FastQC (v0.11.5; Babraham Bioinformatics), aligned to the Af293 reference genome¹³⁷ using the Burrows-Wheeler Aligner (BWA; v0.7.8) mem¹³⁸ and formatted as sorted BAM files using SAMtools (v1.3.1)¹³⁹. SNPs were detected using HaplotypeCaller (v3.6) from the Genome Analysis Toolkit (GATK; v4) package¹⁴⁰ and

repetitive regions identified using RepeatMasker (v4.0.6)¹⁴¹ were excluded. SNPs were filtered out if they had a minimum genotype quality of less than 50 or met at least one of the following parameters: “DP < 10 || MQ < 40.0 || QD < 2.0 || FS > 60.0 || ABHom < 0.9”. SNPs were mapped to genes using VCF-annotator (Broad Institute, US).

2.11.3. Phylogenetic analysis

SNP data were converted into presence/absence of SNP with respect to the reference genome, low confidence SNPs were converted to missing data and the resulting binary data were converted to relaxed interleaved Phylip format. Maximum-likelihood trees were constructed to assess sequence similarity between isolates using RAxML (v8.2.9)¹⁴² and visualised in FigTree (v1.4.4; <http://tree.bio.ed.ac.uk/software/figtree>). Phylogenies were bootstrapped using the rapid bootstrap algorithm with 500 iterations.

2.12. Maximum daily air temperatures on UK sampling dates

The Met Office produces datasets of climate observations recorded in the UK from 1862 to present day that are available for the public to download. The HadUK-Grid dataset contains daily, monthly, seasonal and annual measures of climate variables formatted by administrative regions, UK countries and 1km, 5km, 12km, 25km and 60km grids¹⁴³. The decision was taken to download daily measures of climate variables for 1km grids for which maximum air temperature, minimum air temperature and total precipitation amount were available. As participants were asked not to sample on days when rain was forecast, as this was likely to reduce the sampler’s efficacy, the chosen daily variable was maximum air temperature. Data were downloaded as NetCDF files for the month in which samples were collected and uploaded to the free and open-source geographic information service (GIS) application QGIS¹⁴⁴ as a raster layer, along with sampling locations for the corresponding sampling round. Sample locations were saved as geometric points in an Esri shapefile and sampled from the HADUK-Grid raster layer to give the daily maximum air temperature for a month for each location in the sampling round. Maximum air temperature was then assigned to each sample based on the date it was collected.

2.13. Land cover classifications of UK sampling locations

The 2019 land cover maps (LCM2019) for Great Britain (GB) and Northern Ireland (NI) produced by UK Centre for Ecology & Hydrology (UKCEH) were used to obtain land cover classes for the locations that air and soil samples were collected from¹⁴⁵. LCM2019 represents a set of geospatial land cover datasets produced by classifying satellite images from 2019 to describe the UK land surface, and is one of a series of LCMs produced by UKCEH since 1990. For the purpose of this analysis, LCM2019 was downloaded for GB and NI as 25m rasterised land parcels in GeoTiff format. LCM2019 for GB uses the British National Grid (BNG) coordinate system [EPSG: 27700] and LCM2019 for NI uses the Irish Grid coordinate system [EPSG:29903] and both contain 21 land cover classes¹⁴⁶, which are detailed in Table 2.3. Validation of LCM2019 for GB by comparison to observations derived from the GB countryside survey 2019 data and open-source National Forest Inventory data estimated that LCM2019 is 79.4% accurate in its land cover classification¹⁴⁶.

Land cover class	Land cover description
1	Deciduous woodland
2	Coniferous woodland
3	Arable
4	Improved grassland
5	Neutral grassland
6	Calcareous grassland
7	Acid grassland
8	Fen
9	Heather
10	Heather grassland
11	Bog
12	Inland rock
13	Saltwater
14	Freshwater
15	Supralittoral rock
16	Supralittoral sediment
17	Littoral rock
18	Littoral sediment
19	Saltmarsh
20	Urban
21	Suburban

Table 2.3: Land cover classifications 1-21 and descriptions for UK Centre for Ecology & Hydrology's land cover map 2019.

LCM2019 for GB and NI were uploaded to QGIS as raster layers. The locations in GB that air and soil samples were collected from, originally provided as postcodes by participants, were converted into BNG eastings and northings using an online batch postcode converter tool and NI postcodes were converted into Irish Grid eastings and northings. Duplicate sampling locations were removed and the remaining locations were uploaded to QGIS. Sample locations were saved as geometric points in an Esri shapefile and sampled from the LCM2019 raster layers to give the corresponding land cover classification for each location. As the majority of participants reported collecting samples from their homes or workplaces, it was expected that the majority of locations would be land cover class 20 (urban) or 21 (suburban). As a result, any locations returning land cover classes 1-19 were visually verified and corrected if necessary. For example, the air sample collected from my home address was classified as land cover class 3 (arable) because it is a new-build house built on previous farmland so this was reclassified as 21 (suburban).

2.14. Percentage of arable land cover in 2 km surrounding UK sampling locations

To calculate the percentage of arable land surrounding sampling locations, the LCM2019 for GB and NI were downloaded from UKCEH's website as vector land parcel datasets in GeoPackage format. They were uploaded to QGIS as vector layers, using the BNG system for the GB LCM and Irish Grid system for the NI LCM. Arable land (land cover class 3) was then extracted from the LCMs to produce new layers, which are shown in Figure 2.8. Unique sampling locations for GB and NI, converted to BNG and Irish Grid eastings and northings respectively, were uploaded to QGIS and a 2 km buffer was added to each point. Overlap analysis in QGIS was then used to calculate the percentage of arable land within each 2km buffer.

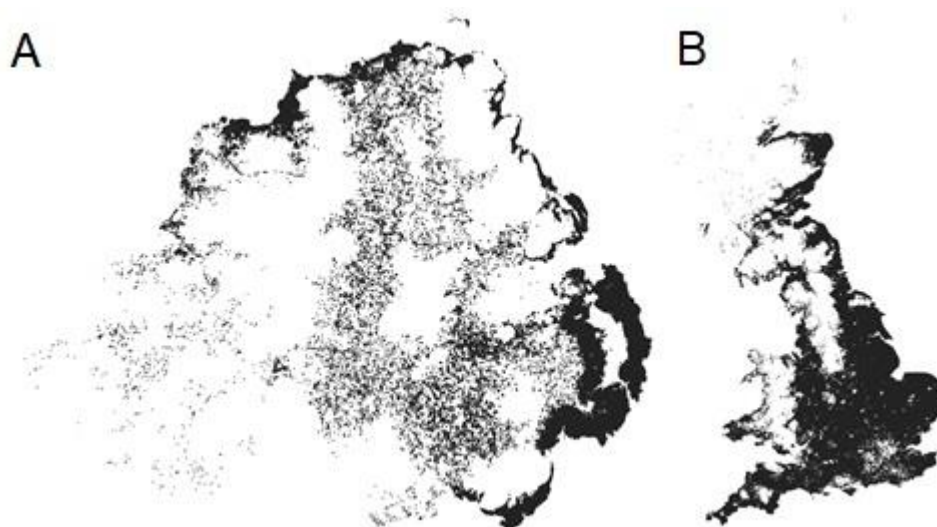


Figure 2.8: Arable land cover in A) Northern Ireland and B) Great Britain shown here in black after land cover class 3 was extracted from the UKCEH 2019 land cover maps.

2.15. Distances from sampling locations to nearest composting facility with open windrow or outdoor activity

2.15.1. In England

The locations of composting facilities in England with open windrow or outside activity (OW/OA) and active permits to operate at the end of 2017 were obtained from the Environment Agency (EA), in agreement with the requirements of their conditional license. The postcodes were converted to latitude and longitude coordinates and BNG eastings and northings using an online batch postcode converter tool and duplicate locations were removed, which gave 208 unique composting site locations. The locations of these sites are shown in green in Figure 2.9A and a screenshot from Google maps of an open windrow composting facility is shown in Figure 2.9B.

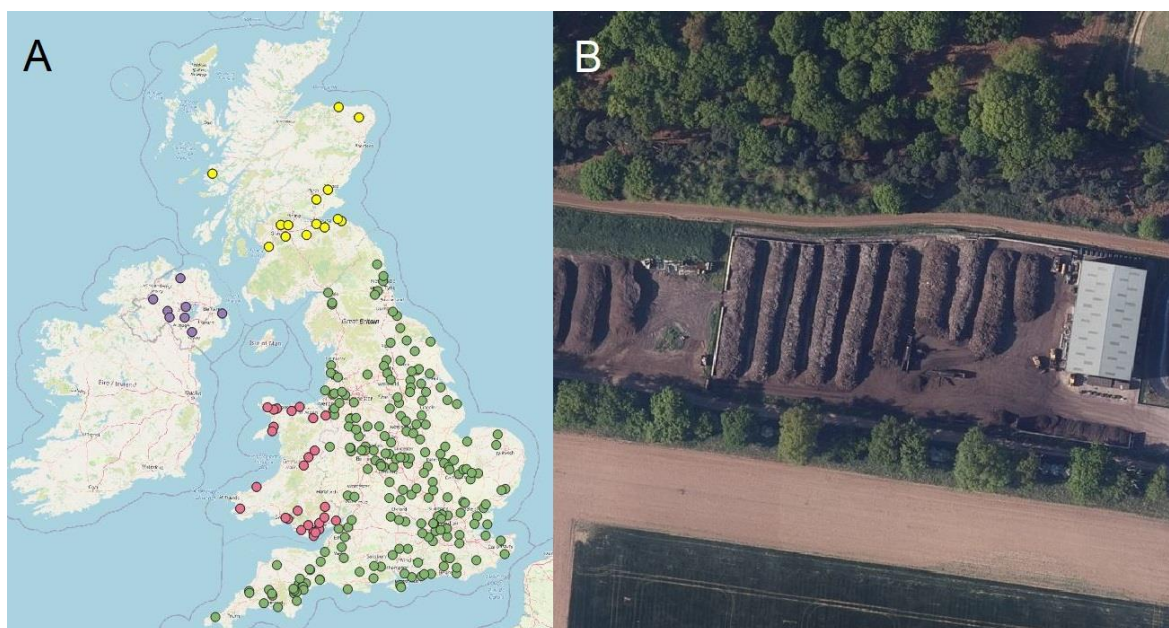


Figure 2.9: A) Map showing the location of composting facilities in the UK with open windrow or outdoor activity: green dots are sites in England, pink in Wales, yellow in Scotland and purple in Northern Ireland, and B) screenshot from Google maps showing an open windrow composting facility.

2.15.2. In Scotland

The locations of waste sites in Scotland were downloaded from the Scottish Environment Protection Agency (SEPA) website¹⁴⁷ and sites were selected that have an active permit to operate and had their waste site activity listed as “composting”. The national grid reference was used to obtain latitudes and longitude coordinates and BNG eastings and northings for each site, and the sites were visualised on Google maps using satellite view to confirm the presence of open windrow or outdoor activity. This gave 14 unique composting sites, the locations of which are shown in yellow in Figure 2.9A.

2.15.3. In Wales

The locations of waste sites in Wales were downloaded from the Natural Resources Wales website¹⁴⁸ and sites were selected that had an active permit to operate at the time of access and the following waste activity codes: “A22: Composting Facility”, “S0816: SR2008 No16 : Composting in open windrows” and “SR/12: SR2010 No12: Treatment of waste to produce soil <75,000 tpa”. The national grid reference was used to obtain latitudes and longitude coordinates and BNG eastings and northings for each site, and the sites were visualised on Google maps using satellite view to confirm the presence of OW/OA. Two sites were excluded

due to lack of evidence of OW/OA, which left 27 unique composting facilities. The locations of these sites are shown in pink in Figure 2.9A.

2.15.4. In Northern Ireland

The locations of waste sites in Northern Ireland that reported composting in their specified operations were downloaded from the Northern Ireland Environment Agency website¹⁴⁹. The postcodes were converted to latitude and longitude coordinates and BNG eastings and northings, and sites were visualised on Google maps using satellite view to confirm the presence of open windrow or outdoor activity. This gave eight unique composting sites, the locations of which are shown in purple in Figure 2.9A.

Altogether there were 257 composting facilities in the UK identified as OW/OA. The package “geosphere” in R version 4.0.0¹⁵⁰ was used to calculate the distance from a) UK sampling locations and b) all UK postcodes to their nearest composting facility.

2.16. Generalised linear models

Generalised linear models (GLMs) were carried out using R version 4.0.0 to look for associations between climate, land use and a) the likelihood of a sample growing *A. fumigatus* or azole-resistant *A. fumigatus* (ARAF), or b) the number of *A. fumigatus* or ARAF colonies grown from a sample. Growth of *A. fumigatus* or ARAF from a sample is a binary (0/1) dependent variable, therefore logistic regressions (“glm” function; family = “binomial”) were carried out to look for associations. The numbers of *A. fumigatus* or ARAF colonies grown from a sample are count data for which Poisson regressions (“glm” function; family = “Poisson”) are usually carried out; however, in the case of overdispersed count data, Quasi-Poisson or negative binomial regressions are used instead. Negative binomial regressions (library “MASS”; “glm.nb” function) allow the dispersion parameter to vary according to the data, instead of setting it to 1 as in Poisson regressions. Independent variables were selected for inclusion in a model based on a significant improvement on the null model, as determined by analysis of variance (ANOVA) using chi-squared test. Results were considered significant when $p \leq 0.05$. The model with the best fit was chosen based on a reduced Akaike information criterion (AIC) score and a significant improvement on the null model.

Chapter 3: Air- and Soil-Sampling Mycology

Citizen Science Projects

A concise version of this chapter has been peer-reviewed and published in the journal Citizen Science: Theory and Practice.

Shelton, J. M. G., Fisher, M. C. & Singer, A. C. Campaign-Based Citizen Science for Environmental Mycology: The Science Solstice and Summer Soil-Stice Projects to Assess Drug Resistance in Air- and Soil-Borne *Aspergillus fumigatus*. *Citiz. Sci. Theory Pract.* **5**, 20 (2020).

DOI: <https://doi.org/10.5334/cstp.325>

Abstract

Citizen science projects have traditionally been undertaken for ecological purposes- to observe phenological changes, wildlife populations or the spread of invasive species- and are now gaining traction in microbiology research to track the emergence and spread of pathogens in the environment.

As previous studies that have collected *Aspergillus fumigatus* spores from the air and soil in the UK have been limited in their geographical reach, a citizen science approach was used in this thesis for sample collection. Volunteers residing in the UK were asked to collect air and soil samples from their homes and workplaces on five solstice and equinox dates between June 2018 and June 2019 and return them to the lab in Freepost envelopes. *A. fumigatus* fungal spores were then cultured from the samples and later tested for susceptibility to azole drugs. Overall, 787 volunteers returned 2,132 air samples and 509 soil samples, which grew a total of 7,991 *A. fumigatus* colonies.

The citizen science approach detailed in this chapter has allowed for UK-wide surveillance of drug-resistant *A. fumigatus* by facilitating the simultaneous collection of hundreds of environmental samples on the same day. This sampling approach was economical, costing approximately £2,850 in total or the equivalent of £1 per sample, and has demonstrated that citizen science participation is valid, and incredibly useful, for mycological research.

3.1. Introduction

3.1.1. History of citizen science

In 2014, the term “citizen science” was added to the Oxford English Dictionary (OED) and defined as “scientific work undertaken by members of the general public, often in collaboration with or under the direction of professional scientists and scientific institutions”. Citizen science has a long history, as before science was professionalised it was undertaken by hobbyists and amateur scientists. An early example in the UK is Robert Marsham who, in 1736, started recording phenological events for plant and animal species on his family estate in Norfolk¹⁵¹. After his death, his work was continued until 1947 by five generations of his family resulting in a 211-year long record of phenological changes¹⁵¹. From 1875 until 1947, the Royal Meteorological Society (RMS) established a national network of volunteers to record phenological data alongside its weather measurements¹⁵². Restarted by the UK Centre for Ecology & Hydrology (UKCEH) in 1998, the UK Phenology Network (UKPN) now has 40,000 registered recorders across the UK and a database of over 3 million records¹⁵³.

3.1.2. Ecological data collection by citizen scientists

The majority of citizen science projects involve collecting ecological data and rely on volunteers to record their observations of a species and submit them by post, online or using a smartphone application. When collected over time, these records can demonstrate changes in species populations and/or distributions that are often a result of human activity leading to habitat destruction, pollution or climate change, and can also flag wildlife disease outbreaks. A few current UK examples include the Big Garden Birdwatch¹⁵⁴, which takes place in January and asks volunteers to record bird species visiting their garden, and the Big Butterfly Count¹⁵⁵ from July to August that asks volunteers to record butterfly and day-flying moth species. The Big Moss Map¹⁵⁶ relies on citizen scientists recording sightings of *Sphagnum* moss in the Peak District and South Pennines to aid peatland conservation, and the Moors for the Future¹⁵⁷ hare survey asks citizen scientists to record hare and rabbit sightings. Ecological data collected by citizen scientists in the UK have led to publications on starling murmurations¹⁵⁸, cuckoo sightings¹⁵⁹, bird migrations¹⁶⁰, hedgehog declines¹⁶¹, the impact of artificial lighting on moth species¹⁶² and the distributions of wasps¹⁶³,

bumblebees¹⁶⁴ and ladybirds¹⁶⁵, to name just a few. Citizen scientists can also report sightings of invasive species, such as the horse-chestnut leaf-mining moth¹⁶⁶, harlequin ladybird and Asian hornet, or toxic algal blooms¹⁶⁷ that have conservation implications for native species.

3.1.3. Recording invisible threats to species and environments

Citizen science projects can also observe changes that are not visible, such as pathogen spread, air pollution, microbial diversity and antimicrobial resistance (AMR). Asking citizen scientists to report, photograph or send in samples of wildlife or plants showing signs of disease have allowed scientists to track the incidence of avian pox, an emerging viral disease, in British tits¹⁶⁸, *Trichomonas gallinae*, an emerging protozoal disease, in British greenfinches and chaffinches¹⁶⁹ and acute oak decline (AOD) in British woodlands¹⁷⁰. A Netherlands-based project asked citizen scientists to measure atmospheric aerosols using a low-cost, mass-producible add-on that turns a smartphone into a spectropolarimetric instrument¹⁷¹, while Open Air Laboratories (OPAL) asked volunteers to record lichen species in English woodlands and used these as a proxy for nitrogenous pollution levels¹⁷². Several global projects have asked citizen scientists to send in swabs that are then analysed for bacterial diversity: of stool for American Gut¹⁷³, of surfaces in their home for Wild Life in Our Homes¹⁷⁴, of their belly button for Belly Button Biodiversity¹⁷⁵ and of their armpit¹⁷⁶. The UK-based Swab & Send is an ongoing, self-funded project that asks volunteers to send in swabs from any object or environment of their choice to identify new antibiotic compounds, in response to the growing threat of AMR to human health¹⁷⁷. Such citizen scientist projects may also play an important role in teaching, for example, microbiology courses in schools and universities. For The Wolbachia Project, high school students and undergraduates in the United States (US) collected insects and tested for the presence *Wolbachia* bacteria in their guts by DNA extraction, polymerase chain reaction (PCR), gel electrophoresis, sending amplicons for sequencing and depositing the sequences in the National Center for Biotechnology Information (NCBI) database¹⁷⁸.

3.1.4. Examples of citizen science in mycology

While the majority of citizen science microbiology projects focus on bacteria, there are also a few related to mycology. Kew's Lost and Found Fungi project asked volunteers to look for a

list of 100 rare fungal species in the UK and has received nearly 1,400 records of these species since 2014¹⁷⁹. The Mushroom Finder smartphone application developed by the Mycology Research Society of the University of Vienna allows citizen scientists to submit images and locations of mushrooms¹⁸⁰, and the Global Sourdough Project identified bacteria and yeasts in sourdough starters sent in by bakers around the world¹⁸¹. In both Slovenia¹⁸² and the UK¹⁸³ citizen scientists were asked to report symptoms of die-back in Ash trees, caused by the fungal pathogen *Hymenoscyphus fraxineus*, with UK participants also asked to tag trees they believed to be resistant to disease.

3.1.5. Environmental sampling for azole-resistant *Aspergillus fumigatus*

Inhaling *A. fumigatus* spores has long been recognised as a cause of hypersensitivity and asthma¹⁸⁴ and a survey of air in rural and urban locations in 1976 sought to monitor seasonal fluctuations in spore counts by air sampling daily for two years at multiple outside locations⁶⁶. Since then, studies monitoring for *A. fumigatus* spores in air samples have mostly focussed on industrial composting sites to inform potential health impacts of bioaerosols released by composting on workers and nearby residents^{62,63,129,185–187}. A comprehensive review of all such studies was undertaken by The Composting Association and Health and Safety Laboratory in 2003¹⁸⁸ and reports have been commissioned by the Environment Agency¹³⁰ and Defra¹⁸⁹ on environmental monitoring of bioaerosols at composting facilities.

Recent studies have focussed on collecting *A. fumigatus* spores from air^{134,190,191}, soil^{131,134,190–192} and plant bulbs¹⁹⁰ across the UK and testing them for resistance to azole drugs. Dunne *et al.* (2017) collected outdoor air samples twice monthly throughout January and February 2014 on a hospital campus in Dublin, Ireland, soil samples from commercial compost and from five sites around the hospital, and swabbed the surface of plant bulbs collected from a local garden centre¹⁹⁰. Bromley *et al.* (2014) collected matched air and soil samples from multiple sites in Manchester city centre and from arable fields outside of Manchester with known azole applications from 2009 to 2011¹⁹¹. Tsitsopoulou *et al.* (2018) collected soil samples from urban and rural locations across South Wales and air samples from three hospital sites from June to November 2015¹³⁴. Van Der Torre *et al.* (2020) collected soil samples by swabbing root vegetables from farms and private gardens in 11 UK counties throughout 2018¹⁹², and

Sewell *et al.* (2019) collected soil samples from rural and urban sites across Southern England from May to July 2018¹³¹. Fraaije *et al.* (2020) collected outdoor air samples in February 2018, May 2018 and May 2019 from Harpenden and soil samples from experimental fields in Hertfordshire and Bedfordshire, as well as commercial wheat fields in Kent, Suffolk, Somerset, Norfolk and Herefordshire⁸⁹. Alshareef and Robson (2014) collected outdoor air samples monthly throughout 2009 and 2010 on the University of Manchester campus to measure viable *A. fumigatus* spores, but did not test these spores for azole-resistance⁶⁵.

Together these studies give valuable insight into how widespread azole-resistant *A. fumigatus* (ARAF) spores are in the UK environment, but are individually restricted in their geographical spread due to samples being collected by the study authors themselves. To overcome this limitation, I organised two citizen science projects: an air sampling project consisting of four sampling rounds and a soil sampling project that took place on a single date. The aims of these projects were threefold:

1. To increase the numbers of samples collected and their spatial coverage in the UK from previous studies
2. To collect samples in different seasons throughout the year to determine the effect of seasonality on spore numbers and ARAF, and
3. To determine whether citizen science sample collection methodologies are viable for mycological research.

By detailing how the citizen science projects were organised and undertaken, I hope that other microbiologists and mycologists might consider using citizen science approaches in their own research.

3.2. Methods

3.2.1. Air sampling trial run

The proposed method for collecting air samples had previously been used by Henk *et al.* (2011) to collect *Penicillium chrysogenum* spores from around the world and determine their genetic population structure¹⁹³. Prior to organising the citizen science project, the method was trialled in two separate locations for different periods of time. A MicroAmp™ clear adhesive film (Applied Biosystems™, UK) was cut in half to produce two “air samplers”,

adhesive putty was attached to the top for securing the samplers to a ground floor windowsill and the cover was removed to expose the sticky face of the film. Air samplers were left exposed outside an office at St Mary's campus of Imperial College London (ICL), Paddington for two hours, four hours and six hours. These samplers were incubated at 43°C (see section 2.3 for details) and grew two, one and three *A. fumigatus* colonies, respectively (Figure 3.1A). This constituted an urban location so was repeated for eight hours at a rural location UKCEH Wallingford in Oxfordshire, where the sampler grew two *A. fumigatus* colonies (Figure 3.1B). These four air sampling trial runs yielded two *A. fumigatus* colonies per plate, on average, and the method was deemed suitable and simplistic enough for citizen science sample collection purposes.

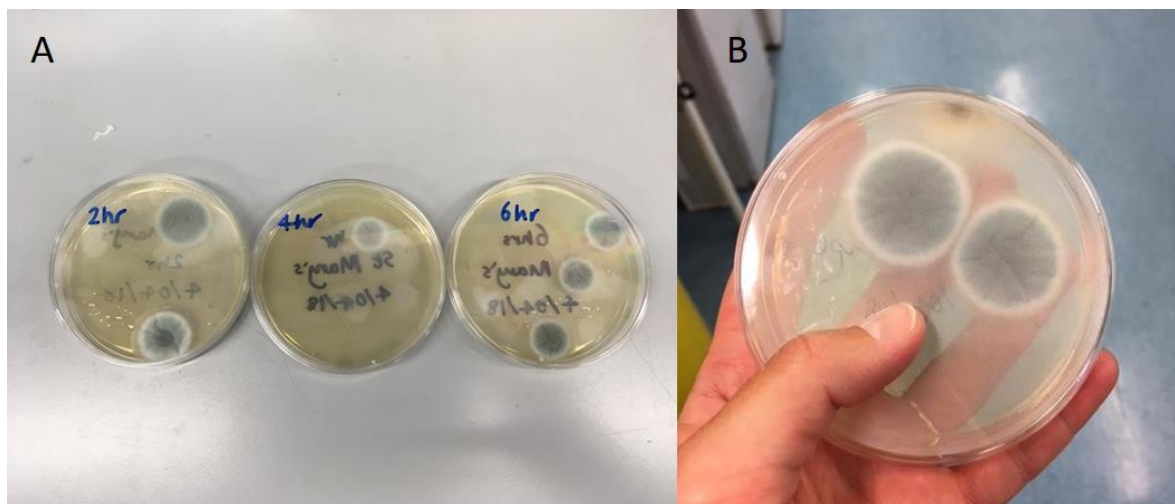


Figure 3.1: A trial run of the air sampling method was undertaken at A) St Mary's campus of Imperial College London, Paddington for two, four and six hours, and B) UKCEH Wallingford, Oxfordshire for eight hours.

3.2.2. Recruitment for citizen science air sampling project

3.2.2.1. Initial air sampling round

Recruitment for a UK-wide citizen science air sampling project to take place on summer solstice, 21st June 2018, began on 30th April 2018. Posts were published on social media platforms Twitter and Facebook containing a poster, a brief description of the project and a link to a Google form (Appendix 3). Posters were displayed on noticeboards at ICL and UKCEH, and supervisors Matthew Fisher and Andrew Singer sent emails to ICL and UKCEH mailing lists, respectively, containing a brief description and a link to the Google form. The project was named "ScienceSolstice" and the poster contained an image of Stonehenge, traditionally associated with solstice activities in the UK, to help participants remember the sampling date.

On the Google form, participants were required to provide their name and address, to receive a sampling pack by post, and their mobile number for a reminder text to be sent the evening before. Optional questions asked them for their email address, the research institute they are affiliated to (if any) and to say how they had heard of the project. All communications were checked for General Data Protection Regulation (GDPR) compliance as of 25th May 2018 and participants were informed *via* the Google form about how their personal data would be used, stored and kept completely confidential. The Google form was closed on 8th June 2018 after 500 participants had signed up to take part in the project.

3.2.2.2. Subsequent air sampling rounds

Due to the success of the air sampling round on summer solstice, it was decided to extend the project to cover solstice and equinox dates in autumn (24th September 2018), winter (21st December 2018) and spring (20th March 2019) of the following year. Due to GDPR restrictions, participants from the first air sampling round could only be contacted again if they had indicated they would like to take part in subsequent rounds. Subsequent air sampling rounds were advertised via posters at ICL and UKCEH and posts on Facebook and Twitter using the names “AutumnAirquinox”, “WinterScienceSolstice” and “SpringAirquinox”, which were successful in re-recruiting many of the participants from the first round plus additional participants. The Google forms for these rounds asked for name, address and email address only.

3.2.3. Participating in citizen science air sampling

Individuals who completed the Google form were sent an air sampling pack containing two air samplers, a poster (Figure 3.2), simple instructions (Appendix 4), a questionnaire (Appendix 5), a brief scientific description of the experimental aims (Appendix 6) and a Freepost return envelope. Participants were asked to secure the air samplers to ground floor windowsills at their home and workplace and expose them for 6-10 hours on 21st June 2018, or a later date if torrential rain was forecast. They were then asked to re-cover the air samplers, complete the questionnaire detailing the exact location the samples were collected from and return both in the Freepost envelope provided. Once received, the air samples were processed in the laboratory as described in section 2.3.

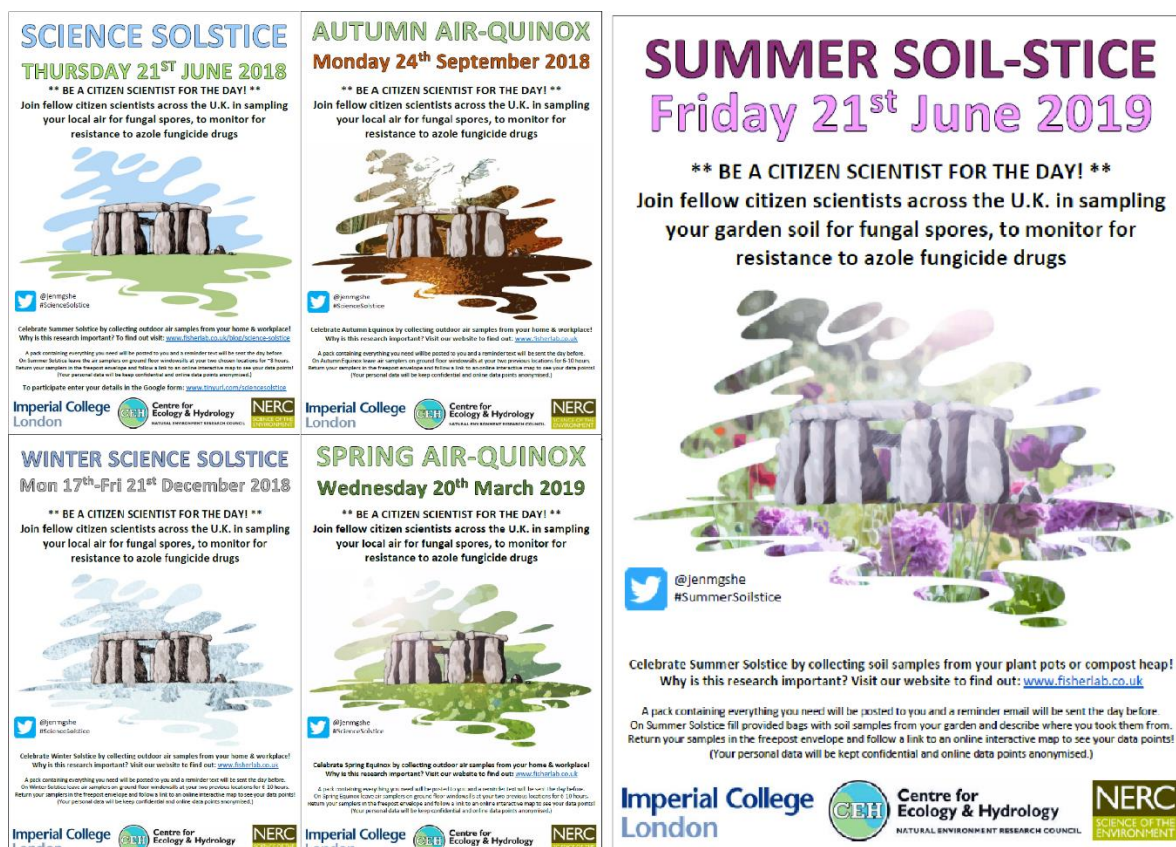


Figure 3.2: Posters advertising the four rounds of the air sampling citizen science project (left panel of four) and soil sampling project (right panel).

3.2.4. Recruitment for citizen science soil sampling project

Once the four rounds of air sampling had taken place, it was decided to organise a final citizen science project asking volunteers to collect soil samples from their garden on summer solstice, 21st June 2019, named "SummerSoilstice". As for the air sampling project, recruitment was *via* posters displayed at ICL and UKCEH and posts on Twitter and Facebook, and participants signed up by entering their name, address and email on a Google form. A blog about the project was also published online on the UKCEH website (Appendix 7). Participants of the second, third and fourth air sampling rounds had been given the opportunity to opt-in to receive project updates, and to provide an email address for further correspondence, and individuals who did so were contacted to ask if they wished to participate in the soil sampling project.

3.2.5. Participating in citizen science soil sampling

Individuals who completed the Google form were sent a soil sampling pack containing two plastic sachets, a wooden spatula, a poster (Figure 3.2), simple instructions (Appendix 8) and a questionnaire (Appendix 9). Participants were asked to collect two soil samples from their garden by using each end of the wooden spatula to scrape surface soil into a plastic sachet. They were also asked to complete the questionnaire detailing the location of their garden and whether the soils were collected from a plant pot, border, compost heap or bag of compost or manure. The questionnaire and soil samples were then returned in the Freepost envelope provided and processed in the laboratory as described in section 2.5.

Participants were advised to exercise caution when sampling from compost heaps and bags of compost or manure as this could lead to aerosolization of a large number of spores, and to only collect samples from their own garden so as to experience similar exposures from sampling as from standard gardening activities such as potting and digging. Participants were asked not to collect samples themselves if they suffer from aspergillosis, were immunosuppressed or had a known lung condition, chronic or acute, as they would be at greater risk of contracting aspergillosis from inhaling large numbers of spores. Individuals were free to opt out at any time by emailing me or by not returning samples.

3.2.6. Citizen science engagement

Participants were encouraged to engage with the projects by taking photos of themselves and their samples and sending the photos by email or posting them on Twitter using the project hashtag. When these photos were provided, participants were asked for their permission to use them in future work and presentations. On the questionnaires provided in each sampling pack, participants were given the option to opt-in to project updates and provide an email address for further communication. If the participant opted in, they were sent an update email 4-6 weeks after each round thanking them for taking part and providing a link to an interactive Google map where they could see the number of *A. fumigatus* isolates their sample(s) grew. Participants were free to follow me on Twitter or follow the project hashtags for further updates.

Four days after the initial air sampling round only, an email was sent to all participants asking for feedback via a different Google form (Appendix 10). The form asked whether the text and email reminders beforehand had been useful, whether the participant would like to take part again if the project was repeated, their reasons for not taking part (if they didn't) and had a comment box for additional feedback.

3.3. Results

3.3.1. Citizen science air sampling

3.3.1.1. In the UK

Across the four air sampling rounds that took place between June 2018 and March 2019, participants in the UK collectively returned 1,894 air samples from all over England, Wales, Scotland and Northern Ireland. In total, 1,293 air sampling packs were sent out and 976 were returned, equating to an overall participation rate of 75%. Table 3.1 shows the breakdown of air sampling packs sent out and returned for each air sampling round, as well as the number of air samples collected. Air samples were collected by 485 unique individuals, who either took part once or in multiple sampling rounds. Of the 356 individuals who took part in the first air sampling round, 160 (43%) also took part in the second round, 120 (32%) took part in rounds one, two and three, and 112 (30%) took part in all four rounds.

Type of sampling	Sampling date	Packs sent out	Packs returned	Return rate (%)	Samples collected
Air	21 st June 2018	461	365	79	712
	24 th September 2018	300	204	68	398
	21 st December 2018	231	165	71	321
	20 th March 2019	301	242	80	463
Soil	21 st June 2019	334	246	74	509

Table 3.1: Numbers of sampling packs sent out and number of packs and samples returned across the four air sampling rounds and one soil sampling round.

Figure 3.3A-D shows screenshots of the Google maps sent out to participants after each air sampling round, with air samples that grew *A. fumigatus* shown in green and those that didn't shown in blue. Figure 3.3F is a UK population density map and shows that the majority of samples were collected from populous areas, with the lowest coverage of samples came from less-populated areas of the UK.

3.3.1.2. Globally

Due to the global nature of Twitter, the air sampling project attracted global participants who between them collected 236 samples. In the first air sampling round, 52 participants collected 144 samples from 16 countries: Australia, Belgium, Canada, Chile, China, France, Germany, Hungary, Italy, Madagascar, New Zealand, Portugal, Spain, The Gambia, the Netherlands and the US. In the second air sampling round, 50 participants collected 92 samples from 7 countries: Canada, France, Germany, New Zealand, Portugal, Spain and the US. For the third and fourth air sampling rounds it was decided not to send any more sampling packs abroad as the Royal Mail Freepost system did not cover return postage of these samples and the global samples were not the focus of this thesis.

3.3.2. My participation in air sampling

On each of the four sampling dates, I shared photos on Twitter of myself participating in the air sampling, which are shown in Figure 3.4A-D. For each round I drove a circular route through Oxfordshire and the Cotswolds exposing air samplers at the same nine locations: 1) my home in Abingdon, 2) the entrance gate at Blenheim Palace, 3) a bus stop at Enstone, 4) a pile of manure near the road leading into Chipping Norton, 5) Chipping Norton town hall, 6) an iron railing at Moreton-in-Marsh cemetery, 7) a bench at Stow-on-the-Wold, 8) an old prison at Northleach, and 9) an iron railing in Burford. The circuit took two hours to complete so I waited six hours before driving the route again to collect the samples to ensure that samples were exposed for a minimum of eight hours. The circular route is shown in Figure 3.4E and the samples I collected are included in the totals in Table 3.1.

3.3.3. Citizen science soil sampling

Soil sampling packs were only sent to UK participants due to restrictions on moving soil samples between countries. As shown in Table 3.1, 334 packs were sent out and 246 were returned resulting in a return rate of 74% and a total of 509 soil samples. Of these 246 participants, 43 (17%) had taken part in one or more of the air sampling rounds. Figure 3.3E shows a screenshot of the Google map sent out to participants after the soil sampling round, with samples that grew *A. fumigatus* shown in green, those that didn't shown in blue and those that were too contaminated with other fungal growth to access the *A. fumigatus* colonies shown in red.

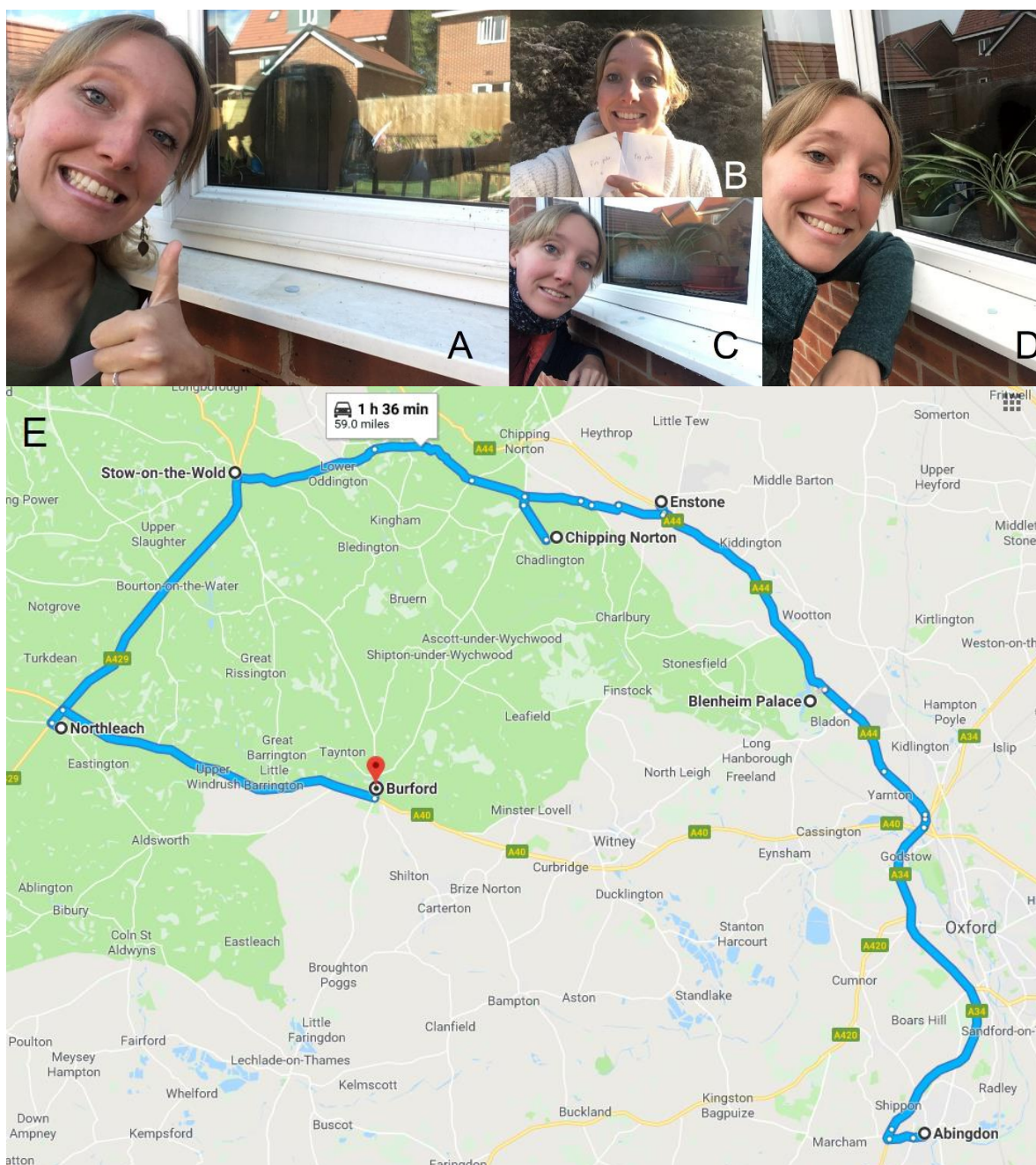


Figure 3.4: Photos of me participating in air sampling on A) 21st June 2018, B) 24th September 2018, C) 21st December 2018 and D) 20th March 2019. E) is a screenshot of a Google map showing the route I drove around Oxfordshire and the Cotswolds on each of the four air sampling dates.

3.3.4. Culturing *A. fumigatus* from samples

The 1,894 air samples collected and returned by UK participants grew 2,366 fungal colonies that were identified macroscopically as *A. fumigatus*, and the 236 air samples collected globally grew 451 *A. fumigatus* colonies. The 509 soil samples grew 5,174 *A. fumigatus* colonies. The numbers of *A. fumigatus* colonies cultured from air and soil samples collected in each sampling round, both in the UK and globally, is shown in Table 3.2.

Type of sample	Sampling date	Number of samples that grew <i>A. fumigatus</i> (% of total) ^a	Number of <i>A. fumigatus</i> colonies cultured ^a
Air	21 st June 2018 <i>UK</i> <i>global</i>	408 (57) 81 (56)	1152 280
	24 th September 2018 <i>UK</i> <i>global</i>	190 (48) 63 (69)	429 171
	21 st December 2018	152 (47)	477
	20 th March 2019	169 (36)	308
Soil	21 st June 2019	327 (64)	5174

Table 3.2: Numbers of samples that grew *A. fumigatus* colonies and the number of colonies cultured across the four air sampling rounds and one soil sampling round. ^a These numbers represent fungal isolates cultured from samples that were identified macroscopically as *A. fumigatus*.

3.3.5. Adherence to sampling date for UK samples

Type of sample	Sampling date	Samples missing date (% of total)	Number of samples collected on intended sampling date (% of total)	Range of sampling dates
Air	21 st June 2018	8 (1)	669 (94)	20 th June – 1 st July
	24 th September 2018	9 (2)	249 (62)	17 th September – 1 st October
	21 st December 2018 ^a	4 (1)	12 (3) ^a	12 th December – 19 th January
	20 th March 2019	5 (1)	287 (61)	13 th March – 24 th April
Soil	21 st June 2019	2 (0)	261 (51)	16 th June – 22 nd July

Table 3.3: Dates that UK samples were collected for four air sampling rounds and one soil sampling round.

^a Due to weather conditions, and proximity to Christmas, participants for the third air sampling round were asked to collect on any suitable day between 17th – 21st December, hence low adherence to sampling date.

Table 3.3 shows the proportion of returned samples that were collected on the intended solstice or equinox date for each sampling round. The first air sampling round had the highest adherence to sampling date (94%) because great emphasis was placed on date for this round, but emails were received on the day from several individuals apologising for not taking part because they were busy. To allow greater flexibility for participants it was decided henceforth to encourage collection of air samples three days either side of the sampling date, hence the drop in adherence to sampling date in subsequent rounds. The third air sampling round had the lowest adherence to date because the winter weather was unpredictable and participants were encouraged to sample on any date between 17th and 21st December, which 274 (85%) did. Timing was less important for soil sampling as this was not affected by weather conditions, therefore less emphasis was placed on sampling date.

3.3.6. Recruitment methods

On the Google sign up form for the first air sampling round participants were asked several optional questions. Of the 513 individuals who completed the form, both in the UK and globally, 233 (45%) indicated they belonged to a research institute and 489 (95%) selected an option for how they'd heard of the project. As expected, the research institutes that accounted for the most participants were UKCEH ($n = 36$) and ICL ($n = 16$). Individuals had heard of the project on Facebook ($n = 139$), by email ($n = 128$), on Twitter ($n = 103$), by word-of-mouth ($n = 97$) and other ($n = 46$). The Tweet recruiting participants for the first air sampling round (#ScienceSolstice) had 642 engagements and made 27,731 impressions, the Tweet recruiting for subsequent air sampling rounds (#AutumnAirquinox, #WinterScienceSolstice and #SpringAirquinox) had 244 engagements and made 13,288 impressions, and the Tweet for soil sampling (#SummerSoilstice) had 823 engagements and made 29,350 impressions.

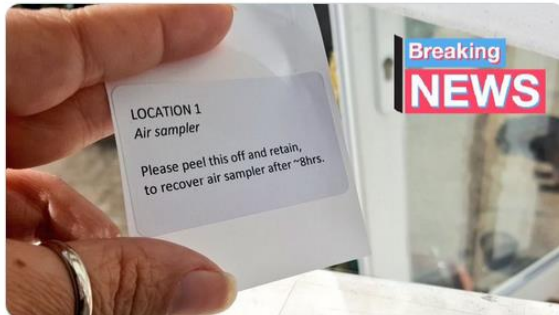
3.3.7. Participant engagement and feedback

Throughout the air and soil sampling projects, participants shared photos on social media and by email of them taking part on sampling days (Figure 3.5). They also sent messages of support by email, on Twitter or handwritten on questionnaires, and I have given talks at several conferences, schools and interest groups upon invitation by participants. I received many emails in response to the project updates congratulating me on the success of the projects, giving words of encouragement and expressing interest and enjoyment at having taken part. Project updates posted on Twitter, often including photos of me preparing sampling packs or processing samples in the laboratory, were well-received and often led to interested individuals getting in touch to participate in an upcoming sampling round.

There were 118 responses to the Google feedback form that was sent out by email after the first air sampling round. Only nine responses were from individuals who had not taken part, for the following reasons: the air sampler(s) blew away or was removed ($n = 6$), the sampling pack did not arrive in time ($n = 1$), and they had not made it to a post box yet but still intended to post ($n = 2$). Of the 118 responders, 115 (98%) found the email reminders ahead of the sampling date useful, 98 (95%) found the text reminder the evening before useful and 115

said they would like to participate again if the project was repeated. 79 responders left additional feedback in the comment box (Appendix 11), which was useful and enjoyable to read.

A fungal [#sciencesolstice](#) begins! Air samplers in place, [@jenmgshe](#)



On my way to pick up the [#ScienceSolstice](#) air sampler from location 2. A beautiful day for [#CitizenScience](#) [@jenmgshe](#)

[@jenmgshe](#) Today is the day. [#ScienceSolstice](#)



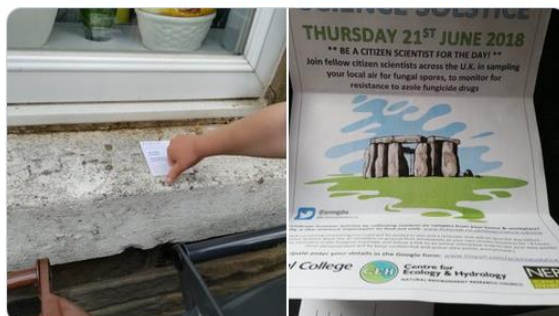
Happy [#ScienceSolstice](#) Day! Air samplers deployed. *Aspergillus fumigatus*, I'm ready for ya! [@jenmgshe](#)



Day 21 of [#30DaysWild](#). Me and the little'n setting up our [#ScienceSolstice](#) spore counter. [@WildSheffield](#) [@DerbysWildlife](#) [@30DaysWild](#) [@jenmgshe](#)



Beautiful day in Cornwall to participate in some [#citizenscience](#). Passive air samplers for fungal spores for [@jenmgshe](#) [#ScienceSolstice](#) [#microbiology](#) project are attached on Penryn Campus...



Completing my [#ScienceSolstice](#) form ready to send in. One sample from my windowsill & one from top of post box. Hope I caught some *Aspergillus fumigatus* spores!! [#WeAreAllCitizenScientists](#) - even those of us who do it for a job! [#CitizenScience](#) [@jenmgshe](#)



Soil samples collected! They are now on their way to [@jenmgshe](#) for her exciting research on azole-resistant fungal spores that can cause aspergillosis disease (or 'fungal asthma'). All the best with the [#ScienceSolstice](#) research Jenny!



Figure 3.5: Several Tweets posted by participants on sampling days showing them taking part in air and soil sampling. (Permission granted by participants for these Tweets to be displayed.)

3.3.8. Amendments to sampling methodology

Feedback from participants on and after air sampling dates led to several modifications being made to air sampling methodologies as the projects progressed. On the day of the first air sampling round, it soon became apparent that several participants had removed the white paper label on the air samples that read “LOCATION 1 (or 2) air sampler: please peel this off and retain, to re-cover air sampler after ~8hrs.” instead of the backing cover (Figure 3.6A), which meant the air samplers were not properly exposed. These participants were contacted immediately to remedy this but 20 air samplers were returned that had not been exposed. It is possible to tell when the air sampler has not been exposed because it remained white and debris-free, whereas exposed samplers were yellow and dusty, and the sticky face and backing cover were perfectly aligned. In subsequent air sampling rounds, the air samplers were instead labelled by hand either “LOCATION 1” or “LOCATION 2” (Figure 3.6B) and participants were referred to the instruction sheet in the sampling pack (Appendix 4) for how to expose the samplers.

Several participants in the first air sampling round reported that adhesive putty was not sufficient for holding the air samplers in place, so for the second sampling round foam adhesive pads were attached to the samplers instead. Participants then reported difficulties in removing these adhesive pads from their windowsills, so for the third and fourth rounds both adhesive putty and foam pads were provided for participants to choose between.

As mentioned in section 3.3.5, several individuals did not take part in the first air sampling round because they were busy on the intended sampling date. As a result of this, in subsequent rounds participants were asked to sample on the sampling date when possible but otherwise within three days either side. A timeline of alterations made to the sampling methodologies is shown in Appendix 12.

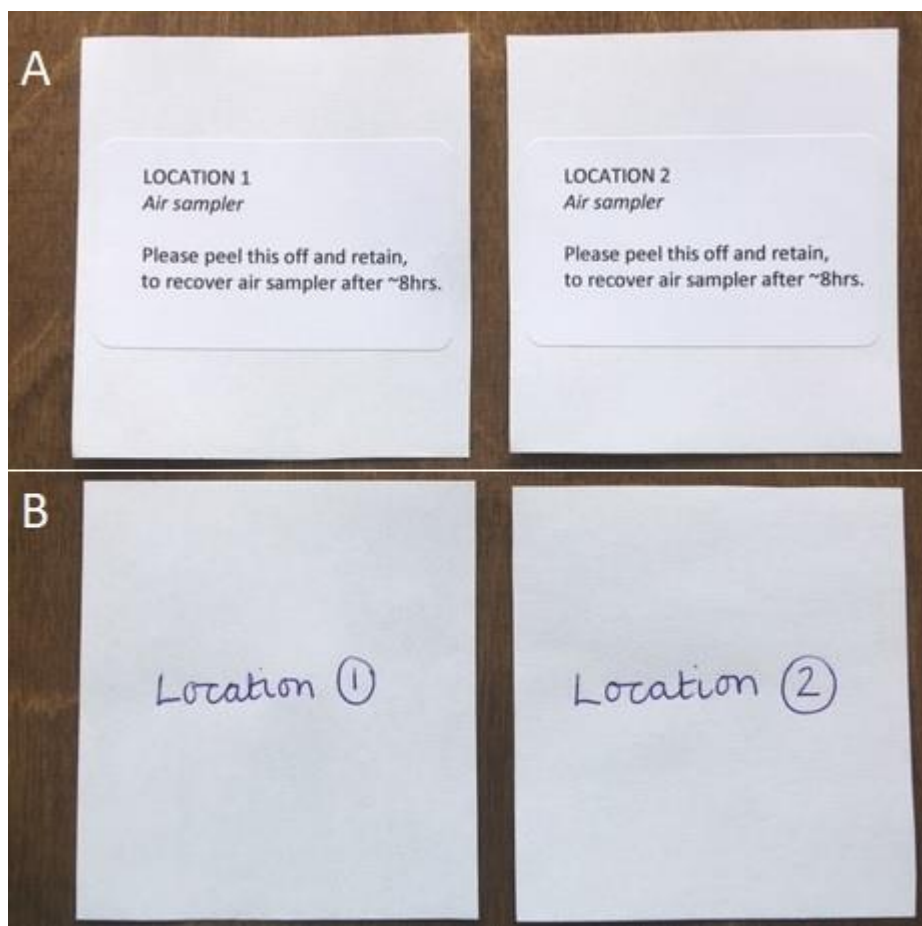


Figure 3.6: A) Air samplers for first air sampling round had sticky labels on the back with location number and basic instructions, but several participants peeled this off instead of the back of the air sampler. B) For subsequent air sampling rounds, location number was handwritten on the back of each air samplers to avoid confusion on how to expose the air sampler.

3.4. Discussion

From June 2018 to June 2019, citizen scientists were asked to collect air and soil samples on five solstice and equinox dates throughout the year and received a total of 2,132 air samples and 509 soil samples from 787 individuals resident in the UK, as well as globally. This achieved a greater sample number and coverage than previous studies that have collected environmental samples in the UK to monitor for *A. fumigatus* and *ARAF*, which was the primary aim of using a citizen science approach in this PhD project. The most localised sample collection previously undertaken was Alshareef and Robson (2014), who collected 24 air samples from 2009 to 2011 outside a university building in Manchester⁶⁵. Dunne *et al.* (2017) collected air and soil samples from a single hospital campus in Dublin from 2014 to 2016 but did not report the total numbers of samples collected¹⁹⁰. Bromley *et al.* (2014) collected air

and soil samples from urban sites in central Manchester and rural areas in Greater Manchester from 2009 to 2011 but also did not report the total numbers of samples collected¹⁹¹. Over 5 months in 2015, Tsitsopoulou *et al.* (2018) collected 671 soil samples from urban and rural locations across South Wales and 44 air samples from three major hospitals in the Cardiff and Newport area¹³⁴. Sewell *et al.* (2019) collected 178 soil samples from four rural locations across Southern England, as well as Bath city centre and multiple sites within London from May to July 2018¹³¹. Fraaije *et al.* (2020) undertook extensive environmental sampling, collecting outdoor air samples between February 2018 and May 2019 from Harpenden and soil samples from urban locations in Hertfordshire, Bedfordshire, Kent, Suffolk, Somerset, Norfolk and Herefordshire, but did not report the total numbers of samples collected⁸⁹. The study with the greatest geographical spread was Van Der Torre *et al.* (2020), who collected 74 soil samples from farms and private gardens located in 11 counties across England throughout 2018¹⁹². To use the same metric for geographical spread as Van Der Torre *et al.* (2020), the 787 individuals who took part in these citizen science projects lived in 47 of the 48 English counties, as well as several locations in Wales, Scotland and Northern Ireland, and 16 other countries.

The secondary aim of these citizen science projects was to collect environmental samples from different seasons throughout the year, to be able to determine whether seasonality has an effect on numbers of *A. fumigatus* or ARAf spores in later analyses. This was successfully achieved for air samples by asking participants to sample on solstice and equinox dates between June 2018 and March 2019 as, despite adherence to sampling date dropping from 94% in the first round to 12-60% for subsequent rounds, the sampling periods did not overlap between sampling rounds so all air samples can be attributed to a different season as intended. It is important to note that the drop in adherence to sampling date was a result of asking participants to sample three days either side of the sampling date, if more convenient to them, because sample number was more important than sampling date. It is also worth noting that the weather on all four air sampling dates was remarkably good in most parts of the UK, which likely increased participation rates and adherence to sampling dates.

The final aim of these projects was to demonstrate that sample collection by citizen scientists is a valid methodology for mycological research, which is evidenced by the number and geographical spread of samples collected and the growth of 7,991 *A. fumigatus* isolates from

all samples combined. This far exceeds the number of isolates collected by previous studies in the UK: for example, 220 isolates from monthly air samples collected in Manchester over two years⁶⁵ and 515 isolates from air and soil samples collected across South Wales over 5 months¹³⁴. It was also reassuring that approximately half of air samples and one-third of soil samples did not grow *A. fumigatus*, which suggests that contamination was not an issue despite citizen scientists not being experienced in sample collection and *A. fumigatus* being a ubiquitous organism. The high return rates of 68-80% seen across the five sampling dates, which are considerably higher than return rates of 10-15% generally expected for citizen science projects (personal comms, CEE Symposium Citizen Science, 14th June 2018), imply that citizen scientists were motivated to take part in these projects. Many participants gave their reasons for taking part in the survey sent out after the first air sampling round, which were enjoyable to read and also gave me valuable feedback on what could be improved for subsequent air sampling rounds.

An unofficial aim of running these projects, because it was not possible to measure its success, was to raise awareness of aspergillosis disease. Early diagnosis and treatment are associated with better patient outcomes¹⁹⁵, yet individuals with aspergillosis reported a delay of up to five years in receiving their diagnosis (personal comms, Sandra Hicks and Gillian Fairweather of The Aspergillosis Trust). Furthermore, a survey of my ~1,200 Twitter followers in April 2020 revealed that only 54% of 1,267 participants had heard of aspergillosis and knew what it was, despite many being scientists or medical doctors. All sampling packs that were sent out contained a two-page description of *A. fumigatus*, aspergillosis and the importance of environmental sampling, and blogs about the projects were published on ICL and UKCEH websites. Articles about the projects have been published in the Botanical Society of Scotland's newsletter and the Society for Applied Microbiology magazine, I have given several conference talks and I have been invited by participants to give talks at schools and public science events. While there is no way to measure the impact of these engagements, my talks have been greeted with interest and enthusiasm and I hope the audience left with a greater understanding of aspergillosis than when they arrived.

Other researchers considering a citizen science approach to data collection might be interested to know that the costs associated with these projects are considerably lower than if I'd attempted to collect the same number and coverage of samples by myself. Recruitment

by social media platforms was free and very effective: the Tweet containing the poster, description and sign-up link for the first air sampling round reached over 27,000 people and the Tweet advertising subsequent air sampling rounds was seen by over 29,000. Stationery was provided by UKCEH and ICL and sampling packs were posted using ICL's franking system. Approximately £800 was spent on setting up and renewing the Royal Mail first-class Freepost return, £1,800 on laboratory reagents and consumables and £50 on additional stationery, bringing the total to £2,850. This is the equivalent of £1 per sample or 33 pence per *A. fumigatus* isolate across the five air and soil sampling rounds.

An aspect of citizen science I had not considered before organising and running the projects was the level of support and encouragement the participants would provide. As soon as I advertised the first air sampling project, individuals were keen to hear more about my research and to participate in sample collection. On sampling dates, individuals engaged with me on social media and *via* email providing photos of them collecting samples and giving feedback on their experience. Every update email received numerous replies from participants expressing their interest in results and/or their enjoyment at being involved, as well as messages of encouragement. Whenever possible I have responded to requests from participants to write articles or to give talks and have therefore interacted with many of these individuals outside of the projects. If my PhD had gone ahead as originally planned - with all samples being collected and processed by myself - it would have been a solitary experience, but as a result of involving citizen scientists I have had the opportunity to interact with hundreds of people and their interest in my research has been a constant morale boost.

To summarise, this citizen science approach has exceeded my expectations in terms of participation rates, number and coverage of samples collected, timing of sample collection and numbers of *A. fumigatus* isolates cultured for onwards analysis. The projects have proved that sample collection by citizen scientists is viable for subsequent mycological research as well as cost-effective, with the added bonus of engaging interested members of the public at a time when increasing emphasis is being placed on science communication. I hope these projects have also increased awareness amongst participants of aspergillosis diseases. I would urge other microbiologists and mycologists to consider using a citizen science approach in their research and to adapt the simple and inexpensive sampling methodologies used here to

monitor environmental levels of other fungal, bacterial or viral pathogens, DNA, toxins, insects or chemicals of interest.

3.5. Acknowledgements

I would like to thank all the individuals who took part in the air and soil sampling citizen science projects: for contributing samples and for their support and enthusiasm throughout. Thank you also to The Aspergillosis Trust and National Aspergillosis Centre for advertising the citizen science projects and recruiting participants, and for their ongoing interest in my research.

Chapter 4: Azole-Resistant *Aspergillus fumigatus* Spores in UK Air

Abstract

Aspergillus fumigatus spores are well-adapted for dispersal on air currents by their small size, hydrophobicity and melanisation of their cell walls that protects them from ultraviolet light. It is their small size and thermotolerance that enables them to establish and grow in human lungs if they are not cleared by the innate immune system. As it is estimated that every human inhales several hundred *A. fumigatus* spores daily, it is important to understand whether these spores have the potential to establish lung infections that are resistant to medical azoles.

In this chapter, 2,366 *A. fumigatus* spores cultured from air samples collected across the UK and Republic of Ireland were tested for susceptibility to the agricultural azole tebuconazole and medical azoles itraconazole, voriconazole, posaconazole and isavuconazole. Of these, 111 isolates (5%) were resistant to tebuconazole, of which 88 isolates (79%) were also resistant to at least one of the medical azoles and 15 (14%) manifested pan-azole resistance to itraconazole, voriconazole, posaconazole and isavuconazole. The *cyp51A* resistance mechanism TR₃₄/L98H was detected in 58 isolates and TR₄₆/Y121F/T289A in six isolates, one isolate contained only TR₃₄, four isolates contained only amino acid substitutions in *cyp51A* and 30 isolates did not contain any *cyp51A* resistance-associated polymorphisms.

Spatial analysis showed no spatial structuring of ARAf across the UK, showing broad exposure of the UK public to this bioaerosol. Variables including season in which the air sample was collected, the proximity of the sample to the nearest composter with open windrow or outdoor activity and the percentage of arable land in the 2 km surrounding sampling location were found to have significant effects on the likelihood of the sample growing *A. fumigatus* and on the number of *A. fumigatus* colonies grown.

These findings highlight a significant public health risk to individuals with pre-disposing factors to aspergillosis in the UK, due to their 1 in 20 chance of inhaling an *A. fumigatus* spore that has acquired azole-resistance. At-risk individuals could be advised not to work or live near to

a composting facility with open windrow or outdoor activity or spend time outdoors when farmers are spreading compost, manure or slurry on their fields, in order to reduce their exposure to airborne *A. fumigatus*. These results also support the need for novel antifungals to treat aspergillosis infections that have acquired pan-azole resistance, despite no prior treatment of the patient with azole drugs, and their ringfencing for medical applications only.

4.1. Introduction

4.1.1. Airborne *Aspergillus fumigatus* spores

Aspergillus fumigatus spores (conidia) are well-adapted to airborne dispersal due to their small size (2.5-3 μm), hydrophobicity, melanin in their cell wall and thermotolerance⁵⁷. It is hypothesised that microbes or organisms smaller than a few millimetres are globally distributed due to their ability to disperse via air currents¹⁹⁶, which has been demonstrated for *A. fumigatus* by the recovery of genetically-similar isolates from clinical and environmental samples across the world^{197,198}. The hydrophobicity of *A. fumigatus* spores is due to the rodlet layer in their cell wall, comprised of low molecular mass secreted proteins called hydrophobins, which repels water¹⁹⁹. As a result, splashing rain has been shown to disperse huge numbers of hydrophobic spores²⁰⁰. The distinctive blue-green colour of *A. fumigatus* spores is produced by the melanin pigment 1,8-dihydroxynaphthalene in the cell wall, which protects the spore from damage caused by ultraviolet (UV) radiation in sunlight as well as chemical stresses, high temperature and host immune responses²⁰¹. *A. fumigatus* is unusually thermotolerant for a eukaryotic organism; it grows rapidly at 37°C, human body temperature, and is one of the few microbes still able to grow at the 50-60°C temperatures found in self-heating compost²⁰². Furthermore, spores can grow in temperatures down to 12°C, can tolerate freezing conditions, and can remain viable after being frozen or dehydrated for many years⁵³. The combination of these factors allows *A. fumigatus* spores to be carried easily by air currents, with Taha *et al.* (2015) showing that a mean wind speed of 1 m/s was sufficient to generate an emission rate of $8\text{--}11 \times 10^3$ colony forming units (CFU)/m²/s of *A. fumigatus* spores from an undisturbed compost pile⁶², whilst remaining unaffected by environmental stresses. Viable *A. fumigatus* spores have even been recovered from the lower atmosphere by flying a drone at 120 m that was customised to hold spore traps²⁰³.

4.1.2. Air sampling methodologies

4.1.2.1. Culture-based

The standard method for detecting airborne microbes is by impaction onto plates containing agar; either by using gravity to deposit onto settle plates (passive air sampling) or by using an air sampling device to draw in air of a pre-selected volume and/or at a fixed rate onto the plate (active air sampling). When culturing fungal spores, the agar can be supplemented with antibiotics to prevent bacterial contamination, growth-suppressants such as Rose Bengal to prevent contamination by fast-growing mucoraceous moulds, or antifungals to select for drug-resistant spores⁵⁷. The type of agar used can also be varied to target different fungal species. The plates are then incubated for several days - duration and temperature dependant on the target species - and CFUs determined based on macroscopic and microscopic appearance of colonies. Settle plates are an inexpensive method for detecting whether fungal spores are present, for example as contaminants in pharmaceutical clean rooms or in hospital theatres, and results are expressed as CFU/plate/time²⁰⁴. As air sampling devices record the volume of air impacted on the plate, the CFU/m³ can be calculated from active air samples⁵⁷ but the cost of the device can be a limiting factor for those wishing to undertake air sampling. The limitations of culture-based methods are a) the time taken to culture fungi, b) the risk of contamination, and c) that not all fungal species can be grown in culture.

4.1.2.2. PCR-based

Other active air samplers impact air onto adhesive plastic tape attached to a rotating drum (7-day volumetric spore trap, Burkard Manufacturing Co Ltd, Hertfordshire, UK) or collect air into Eppendorf tubes (cyclone sampler for airborne particles, Burkard Manufacturing Co Ltd, Hertfordshire, UK), and each length of tape or tube corresponds to a duration of time and/or volume of air drawn into the sampler. Fungal spores are recovered by suspension of the tape or tube in solution, DNA is extracted and PCR is performed: either using primers specific for a particular fungal species²⁰⁵ or universal primers for profiling fungal diversity²⁰⁶. The limitations of PCR-based methods are that a) presence of fungal DNA does not inform whether the airborne spore was viable, b) primers are biased towards amplification of different fungal groups, and c) identification is reliant on the fungal sequence being present in the reference database.

4.1.3. Air sampling for *A. fumigatus* in the UK

4.1.3.1. Industrial composters

The composting process has long been associated with high levels of *A. fumigatus*²⁰⁷, with mechanical agitation of open-air compost piles highlighted as the greatest emission source of airborne spores from composting sites to the surrounding environment^{208,209}. As a result of this, numerous studies have been undertaken to assess levels of *A. fumigatus* spores upwind from, at and downwind from industrial composters around the world^{58–60,210,211}. These studies are important to monitor the exposure of workers to *A. fumigatus* spores and other bioaerosols including bacteria, viruses and endotoxins⁶⁹, which have been associated with adverse health outcomes such as increased phlegm production, frequent coughing, colds, bronchitis and shortness of breath²¹². There is also concern for nearby residents after an asthmatic man living in the vicinity of a municipal compost site developed allergic bronchopulmonary aspergillosis (ABPA)²¹³.

In the Department for Environment, Food & Rural Affairs (Defra) 2011 Review of Waste Policy in England, it was estimated that 0.4-0.7 tonnes of carbon emissions could be reduced by diverting food waste from landfill to composting²¹⁴ and as a result the number of large-scale composting facilities is increasing²¹⁵. Open windrow composting is when organic waste is shredded, mixed and placed in uncovered rows that are turned regularly during the 16-week composting process to distribute heat and moisture and improve oxygenation of the waste. This method predominates in the UK and accounted for 90% of total composting undertaken in 2007²¹⁶. Subsequently, there are numerous UK-based studies monitoring release of *A. fumigatus* spores and other bioaerosols from composting facilities^{61–63,129,185–187}, which has led to the Environment Agency issuing the M9 protocol (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/730226/M9_Environmental_monitoring_of_bioaerosols_at_regulated_facilities.pdf) for the environmental monitoring of bioaerosols at these sites. The Environment Agency requires composters with workplaces and dwellings within 250 m of their facility to demonstrate that bioaerosols in this range are at acceptable concentrations above upwind background concentrations, which for *A. fumigatus* spores is 500 CFU/m³ above background⁶⁴. Williams *et al.* (2019) used atmospheric dispersion modelling to demonstrate that concentrations of *A. fumigatus* spores reduced rapidly with distance from composting

facility, with the steepest drop-off between 0 m and 250 m and concentrations reaching background levels at approximately 2 km²¹².

4.1.3.2. Hospitals

Several studies undertaken in the UK and Republic of Ireland to monitor levels of *A. fumigatus* spores have taken place in hospital grounds, due to the risk of immunocompromised patients contracting aspergillosis. Dunne *et al.* (2017) used a dual-head surface air sampler (VWR International, Milan, Italy) to collect twice monthly samples from clinical and nonclinical areas on a hospital campus in Dublin but do not report how many colonies grew from these samples¹⁹⁰. Mullins *et al.* (1984) used Andersen samplers²¹⁷ to collect *A. fumigatus* spores onto malt extract agar (MEA) plates hourly for a year from the roof of a Cardiff hospital and reported an average spore concentration of 11.3 CFU/m³²¹⁸. Tsitsopoulou *et al.* (2018) used a single-headed SAS air sampler (Cherwell Laboratories, Bicester, UK) to collect air samples onto Sabouraud dextrose agar (SDA) plates from clinical sites of three major hospitals in the Cardiff and Newport area in South Wales¹³⁴. Of the 44 air samples they collected, 17 (38.6%) grew *A. fumigatus* colonies.

There is an association between aspergillosis and construction work on or near hospital grounds, unfortunately proven by previous outbreaks^{219–221}. For this reason, Goodley *et al.* (1994) used an SAS air sampler (Cherwell Laboratories, Bicester, UK) to collect weekly air samplers onto SDA plates from three external sites at Guys hospital in London for a year while building work was taking place but did not find an increase in spore levels due to building activity²²².

4.1.3.3. Background levels

Fewer studies have looked at the background levels of *A. fumigatus* spores in UK air that is not near to composting facilities or hospitals. Alshareef and Robson (2014) used an SAS air sampler (Cherwell Laboratories, Bicester, UK) to collect air samples onto potato dextrose agar (PDA) plates monthly for two years from a single location on the University of Manchester campus and reported *A. fumigatus* concentrations between 3 and 20 CFU/m³⁶⁵. Bromley *et al.* (2014) used single- and double-headed SAS air samplers (Cherwell Laboratories, Bicester, UK) to collect air samples onto MEA plates from multiple urban and rural sites around Greater Manchester but do not report how many colonies grew from these samples¹⁹¹. Mullins *et al.* (1976) used MEA settle plates and Anderson samplers to collect weekly samples from two

outdoor urban and two rural locations around Cardiff and report average spore concentrations of 15 CFU/m³⁶⁶. Fraaije *et al.* (2020) used mobile spin air samplers (IUL, Spain) to collect air samples onto SDA plates in Boskoop and Boxtel in the Netherlands and Harpenden in the UK at three timepoints over a 16 month period⁸⁹. They report a daily intake of between 14 and 193 *A. fumigatus* spores at the Boxtel location, based on an adult air intake of 14,000 L.

4.1.4. Seasonal differences in levels of airborne *A. fumigatus* spores

Two monitoring networks have been established in the UK to measure seasonal trends in airborne allergens pollen and fungal spores, including *A. fumigatus*: the Midlands Asthma & Allergy Research Association (MAARA) in Derby established in 1968²²³ and the National Pollen and Aerobiology Research Unit at the University of Worcester established in 2006²²⁴. Both networks use seven-day volumetric spore traps (Burkard Manufacturing Co Ltd, Hertfordshire, UK) and group *Aspergillus* and *Penicillium* species based on their visual similarity under the microscope. MAARA used *Aspergillus/Penicillium* spore numbers recorded from 1970 to 2003 to show that concentrations peaked in the autumn²²⁵, visualised for 1991 to 2003 in Figure 4.1, which agrees with the Worcester station that showed spore numbers to be at their lowest in May increasing to their highest levels in October²²⁴.

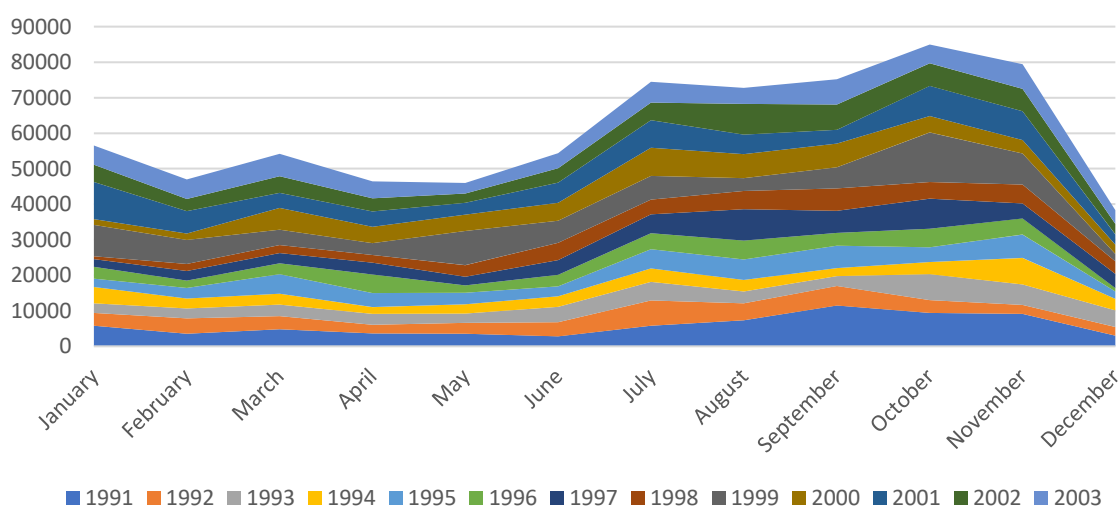


Figure 4.1: *Aspergillus/Penicillium* spore concentrations in outdoor air samples collected in Derby by the Midlands Asthma & Allergy Research Association (MAARA) from 1991 to 2003. Produced from data reported in Millington *et al.* (2005)²²⁵.

Jenkins *et al.* (1980) found a sharp increase in spore counts of *Fungi imperfecti*, which included *Aspergillus* species, in July and August²²⁶ whereas Alshareef and Robson (2014) did not find any significant seasonal variation or correlation with temperature in the number of viable *A. fumigatus* spores collected throughout their 2-year study in Manchester⁶⁵. Using passive air samplers, Mullins *et al.* (1976) found the highest numbers of *A. fumigatus* spores in December and January and the lowest numbers between April and November⁶⁶. Using active samplers, the same study found a peak in spore numbers in March and the lowest numbers between May and October. All studies showed that *Aspergillus* species or *A. fumigatus* spores were present in air samples all year round.

The peak of asthma hospital admissions and deaths in the UK occurs in late summer to autumn^{227–229} and it is likely that exposure to increased levels of mould spores in outdoor air contributes to this. Targonski *et al.* (1995) found that the odds of asthma-related mortality in 5- to 34-year-olds in Chicago were 2.16 times higher on days that mould spore counts exceeded 1000 spores/m³ compared to days when mould spore counts were below this threshold²³⁰. Research is ongoing to define the link between outdoor and indoor spore levels, “Sick Building Syndrome” and asthma-related illness and mortality.

4.1.5. Background levels of azole-resistant airborne *A. fumigatus* spores

It is currently not possible to estimate the proportion of airborne *A. fumigatus* spores circulating in the UK that are azole-resistant because of the limited geographical spread of existing studies and/or lack of information provided to calculate this. Tsitsopoulou *et al.* (2018) collected 44 air samples from outside three major hospitals in Cardiff and Newport that grew 17 *A. fumigatus* isolates, all of which were susceptible to azoles¹³⁴. Dunne *et al.* (2017) do not disclose how many air samples they collected from outside the hospital in Dublin, nor how many *A. fumigatus* isolates grew from them, only that two of the isolates were azole-resistant and contained TR₃₄/L98H¹⁹⁰. Bromley *et al.* (2014) collected an unknown number of air samples from urban areas in Manchester and 150 air samples from rural areas, which grew an undisclosed number of *A. fumigatus* isolates, of which one isolate was azole-resistant and contained the following amino acid substitutions in cyp51A: D100G, V101I and N102H¹⁹¹. Fraaije *et al.* (2020) do not disclose how many air samples they collected from

Harpenden or how many *A. fumigatus* isolates grew from them, only that two isolates were azole-resistant and one of these contained TR₃₄/L98H⁸⁹.

In order to identify locations or activities in the UK associated with higher outdoor concentrations of airborne *A. fumigatus* or ARAf spores than 'background', it is important to define the baseline concentrations of spores and how they naturally fluctuate between seasons and rural and urban areas. The challenges for doing so currently are: 1) the lack of studies characterising baseline concentrations of *A. fumigatus* or ARAf spores in the UK, 2) existing UK studies lack the duration or geographical spread of sampling to compare spore concentrations between seasons and areas, and 3) inconsistency in the way that spore concentrations are reported between studies. The aims of this chapter were fourfold:

1. To ascertain baseline concentrations of airborne *A. fumigatus* and ARAf spores in the UK,
2. To establish whether airborne ARAf spores in the UK have cross-resistance to agricultural and medical azoles,
3. To characterise *cyp51A* polymorphisms in airborne ARAf spores, and
4. To determine whether environmental variables influence spore numbers or azole-resistance in the air.

These aims were achieved by culturing *A. fumigatus* from the air samples collected by citizen scientists on four dates throughout the year from locations across the UK (Chapter 3).

4.2. Methods

4.2.1. Air sampling methodology

The logistics of a citizen science approach to air sampling meant that the sampling methodology needed to be compact, low-cost and simplistic. It was never feasible to supply participants with an active air sampler, which meant that sample collection had to be done using a passive method. Settle plates containing MEA, PDA or SDA nutrient media were considered but problems included: a) restrictions on posting liquids using Royal Mail, b) growth of microorganisms on agar after collection and in transit having potential health impacts for citizen scientists or postal workers, and c) increased cost of Freepost return for

large letters as opposed to standard letters. By comparison, spores on adhesive films are unable to germinate and grow until they were placed on growth medium.

The chosen method of exposing the sticky side of an adhesive film to collect spores had already been used by Henk *et al.* (2011) for collecting *Penicillium chrysogenum* spores¹⁹³ and it was demonstrated to also work for collecting *A. fumigatus* spores in urban and rural locations (section 3.2.1). Each air sampler was compact (6.8 cm x 8.0 cm), lightweight (2 g), easy to attach in place using adhesive putty or foam adhesives, straightforward to expose by peeling off the backing cover, only cost 25 pence and two samplers together were suitable for posting in a Royal Mail standard letter.

4.2.2. Processing air samplers, culturing *A. fumigatus* and testing for ARAf

When sampling packs were received back from citizen scientists, the air samplers and paperwork were processed as detailed in section 2.3. Briefly, the cover of the air sampler was removed and the sticky side was placed face-down on an SDA plate containing penicillin (200 mg/L) and streptomycin (400 mg/L). The plate was incubated for 24 hours at 43°C, the sampler was removed and the plate was incubated for a further 24 hours at 43°C. *A. fumigatus* colonies were picked into a tube containing mould preservation solution (MPS), which was labelled and refrigerated. Each *A. fumigatus* isolate was then tested for susceptibility to tebuconazole (6 mg/L), as detailed in section 2.4.

4.2.3. Cross-resistance to medical azoles

Isolates found to be resistant to tebuconazole were taken to the Public Health England (PHE) Mycology Reference Laboratory in Bristol for antifungal drug susceptibility testing to the four medical azoles itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ), using the Clinical and Laboratory Standards Institute (CLSI) methodology as detailed in section 2.8. Resistance to ITZ, VCZ and ISZ was considered as an MIC $\geq$ 2 mg/L and resistance to PCZ was considered as an MIC $\geq$ 0.5 mg/L.

4.2.4. Cryopreservation, DNA extraction and gene sequencing of ARAf

Isolates found to be tebuconazole-resistant were cryopreserved at -80°C (section 2.6) and had their DNA extracted and stored at -20°C (section 2.7). The DNA from these isolates was then amplified and sequenced for the *cyp51A* gene promoter and coding regions to determine whether the known resistance-associated *cyp51A* polymorphisms were present (section 2.9).

4.2.5. Confirming identity as *A. fumigatus*

Isolates were picked from SDA plates and preserved based on their macroscopic appearance as *A. fumigatus*. If an isolate looked or behaved differently when subsequently cultured, or failed to amplify with the *cyp51A* primers, its identity as *A. fumigatus* was assessed by its microscopic appearance, using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and/or beta-tubulin gene sequencing, as detailed in section 2.10.

4.2.6. Whole-genome sequencing

Tebuconazole-resistant isolates collected in the first air sampling round (prefix SS18) were sent for whole-genome sequencing (WGS). High quality genomic DNA (gDNA) was DNA extracted, purified and sequenced as detailed in section 2.11.1. The raw WGS reads were processed (section 2.11.2) and a phylogenetic tree was produced (section 2.11.3) that included 37 tebuconazole-resistant SS18 *A. fumigatus* UK isolates, eight tebuconazole-resistant SS18 *A. fumigatus* global isolates and 212 additional UK *A. fumigatus* isolates from Rhodes *et al.* (2021)²³¹. Of these 212 isolates, 147 were clinical and 65 were environmental and all were collected in England, Wales, Scotland and Republic of Ireland between 2005 and 2017.

4.2.7. Environmental factors relating to UK sampling dates and locations

To assess the impact of climate and land use variables on the growth and numbers of *A. fumigatus* and ARAf spores, additional data were obtained for each sample according to its

location and date it was collected. Maximum daily temperatures were obtained for sampling dates, as detailed in section 2.12, to determine whether ambient temperature affected the viability of *A. fumigatus* or ARAf spores. The land cover classification of each sampling location and the percentage of arable land in the 2 km buffer surrounding it was obtained from UK Centre for Ecology & Hydrology (UKCEH) land cover map for 2019 (LCM2019), as detailed in sections 2.13 and 2.14, and the distance from sampling locations in England ($n = 1,502$) to their nearest composting facility with open windrow or outdoor activity (OW/OA) was calculated, as detailed in 2.15. These variables were to determine whether land use at or surrounding the sampling location, or its proximity to an OW/OA composter, affected whether the sample grew *A. fumigatus* or ARAf or the number of *A. fumigatus* or ARAf colonies that grew.

4.2.8. Generalised linear models

	Name of variable	Type of data	Number of samples included
<i>Dependent variables</i>	Growth of <i>A. fumigatus</i>	Binary	1,894
	Number of <i>A. fumigatus</i> grown	Count	918 ^a
	Growth of ARAf	Binary	1,894
	Number of ARAf grown	Count	73 ^a
<i>Independent variables</i>	Sampling round	Categorical	1,894
	Date sample collected	Categorical	1,894
	Maximum daily temperature at sampling location	Continuous	1,889 ^b
	Land cover classification of sampling location	Categorical	1,889 ^b
	Urban or rural sampling location	Categorical	1,889 ^b
	Percentage arable land in 2 km buffer surrounding sampling location	Continuous	1,889 ^b
	Distance to nearest OW/OA composter	Continuous	1,889 ^b
	Land cover classification of nearest OW/OA composter	Categorical	1,889 ^b
	Urban or rural location of nearest OW/OA composter	Categorical	1,889 ^b

Table 4.1: Dependent and independent variables used in generalised linear model fitting to determine the influence of climate and location on growth of *A. fumigatus* and azole-resistant *A. fumigatus*. ^a Counts of 0 were excluded to avoid replicating the logistic regressions. ^b Data available for samples collected in England, Wales, Scotland and Northern Ireland hence samples collected from the Republic of Ireland were not included. OW/OA composter = composter with open windrow or outdoor activity.

The dependent and independent variables used in fitting the generalised linear models (GLMs) are shown in Table 4.1. Logistic regressions were used to look for associations

between these independent variables and the likelihood of a sample growing *A. fumigatus* or ARAf. Poisson regressions were deemed unsuitable for analysis due to overdispersion of the count data, shown in Figure 4.2, which resulted in the residual deviance in the model being far greater than degrees of freedom when these should be in approximately 1:1 ratio. Negative binomial regressions were used instead to determine whether any of the independent variables significantly affected the number of *A. fumigatus* or ARAf colonies grown from a sample.

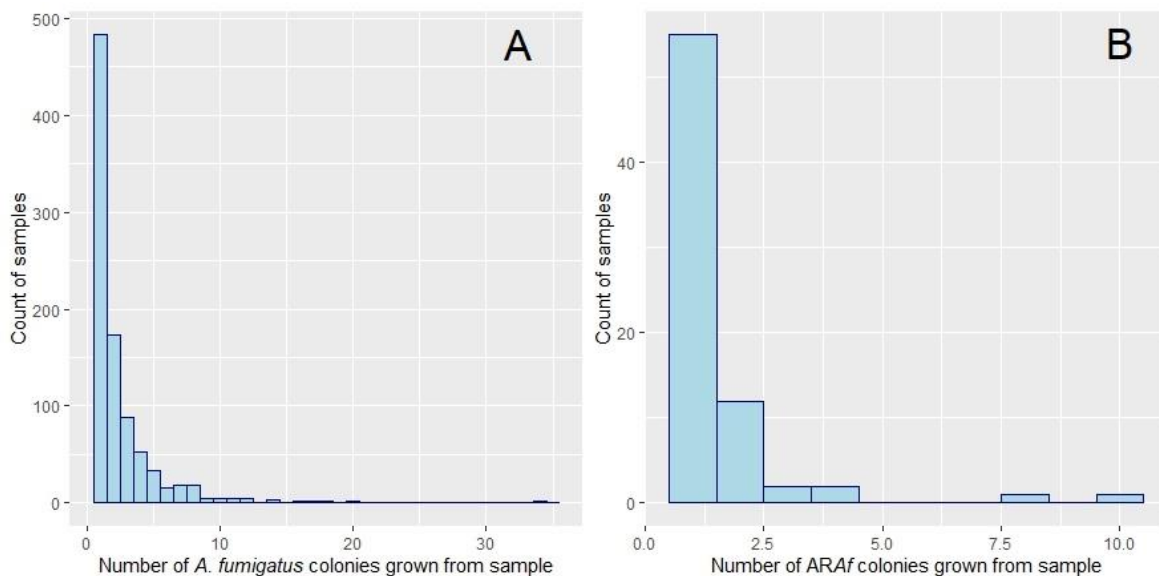


Figure 4.2: Histograms showing the distribution of numbers of A) *A. fumigatus* colonies and B) azole-resistant *A. fumigatus* colonies grown from positive samples.

4.2.9. Geographically weighted regression

Analysis using geographically weighted regression (GWR) was carried out using ArcGIS Pro version 2.7.2²³². GWR allows the relationship between the dependent and independent variables to vary in space, which it does by calculating a regression equation for each location and comparing to regression equations for a chosen number of neighbouring locations. To perform this analysis, the models of best fit chosen by the GLMs were run using GWR including the latitude and longitude of each sampling location. As GWRs only accept continuous independent variables, maximum daily temperature, percentage arable land in 2 km surrounding buffer and distance to nearest composter were used in the analysis. To account for effect of season, data were subset by sampling round and a GWR was run on each subset separately. Currently, GWR can only be run on data with a Gaussian, binomial or

Poisson distribution; therefore, GWRs were run to determine whether location affected the likelihood of a sample growing *A. fumigatus*. GWRs that were run to determine whether location affected the likelihood of a sample growing ARAf failed due to insufficient numbers of positives. The GWRs output regression coefficients and standard errors for the relationship between the dependent and independent variables at each sampling location, which were converted into odds ratios and 95% confidence intervals (CIs). A relationship between variables was considered significant if the 95% CIs were both less than or both greater than one.

4.3. Results

4.3.1. Validation of air sampling methodology

As discussed in Chapter 3, the total number of UK air samples returned ($n = 1,894$) and the return rates of 68-80% across all four air sampling rounds demonstrate that the air sampling methodology used was straightforward enough for individuals to use without training. Furthermore, the growth of 2,366 *A. fumigatus* isolates from air samples collected in the UK and 451 isolates from those collected globally confirmed that the collection and temporary storage of spores on adhesive films did not affect their viability.

An additional consideration for using adhesive films instead of settle plates was that a proportion of air samples (not recorded but estimated at 5%) grew mucoraceous moulds. Like *A. fumigatus*, these moulds grew at 43°C, were often tebuconazole-resistant and had the potential to be pathogenic. If settle plates containing nutrient media were used, it is likely that both *A. fumigatus* and the mucoraceous moulds would have germinated and started to sporulate immediately after collection. All sample processing and subsequent handling of cultures was done in a category 2 hood whilst wearing personal protective equipment (PPE) in recognition of the pathogenic potential of *A. fumigatus* and environmental contaminants (section 2.1).

4.3.2. ARAf in UK air samples

There were 1,894 air samples collected by UK citizen scientists across the four air sampling dates and 919 (49%) samples grew a total of 2,366 *A. fumigatus* colonies. Of the samples that

grew *A. fumigatus*, 73 (8%) samples grew 111 colonies that were resistant to tebuconazole at a concentration of 6 mg/L: of which 42 (38%) were collected in the summer, 26 (23%) in autumn, 15 (14%) in winter and 28 (25%) in spring sampling rounds. The breakdown of air samples collected and those that grew *A. fumigatus* and ARAf by sampling date is shown in Table 4.2, and the numbers of *A. fumigatus* and ARAf colonies grown for each sampling date is shown in Table 4.3. Figure 4.3 shows the locations of samples collected across the four sampling dates, with samples that did not grow *A. fumigatus* shown in blue, samples that grew *A. fumigatus* shown in green and samples that grew ARAf shown in red.

Sampling date	Number of air samples collected	Number of air samples that grew <i>A. fumigatus</i> (% of samples)	Number of air samples that grew ARAf (% of samples that grew <i>A. fumigatus</i>)
21 st June 2018	712	408 (57)	30 (7)
24 th September 2018	398	190 (48)	14 (7)
21 st December 2018	321	152 (47)	12 (8)
20 th March 2019	463	169 (37)	17 (10)
Total:	1,894	919 (49)	73 (8)

Table 4.2: The number of air samples collected, the number of air samples that grew *A. fumigatus* and the number of samples that grew azole-resistant *A. fumigatus* across the four air sampling dates.

Sampling date	Number of <i>A. fumigatus</i> isolates grown from air samples	Average number of <i>A. fumigatus</i> isolates grown per air sample	Number of ARAf isolates (% of <i>A. fumigatus</i>)
21 st June 2018	1,152	2.8	42 (4)
24 th September 2018	429	2.3	26 (6)
21 st December 2018	477	3.1	15 (3)
20 th March 2019	308	1.8	28 (9)
Total:	2,366	2.6	111 (5)

Table 4.3: The number of *A. fumigatus* and azole-resistant *A. fumigatus* isolates grown from air samples and the average number of *A. fumigatus* isolates grown per sample across the four air sampling dates.

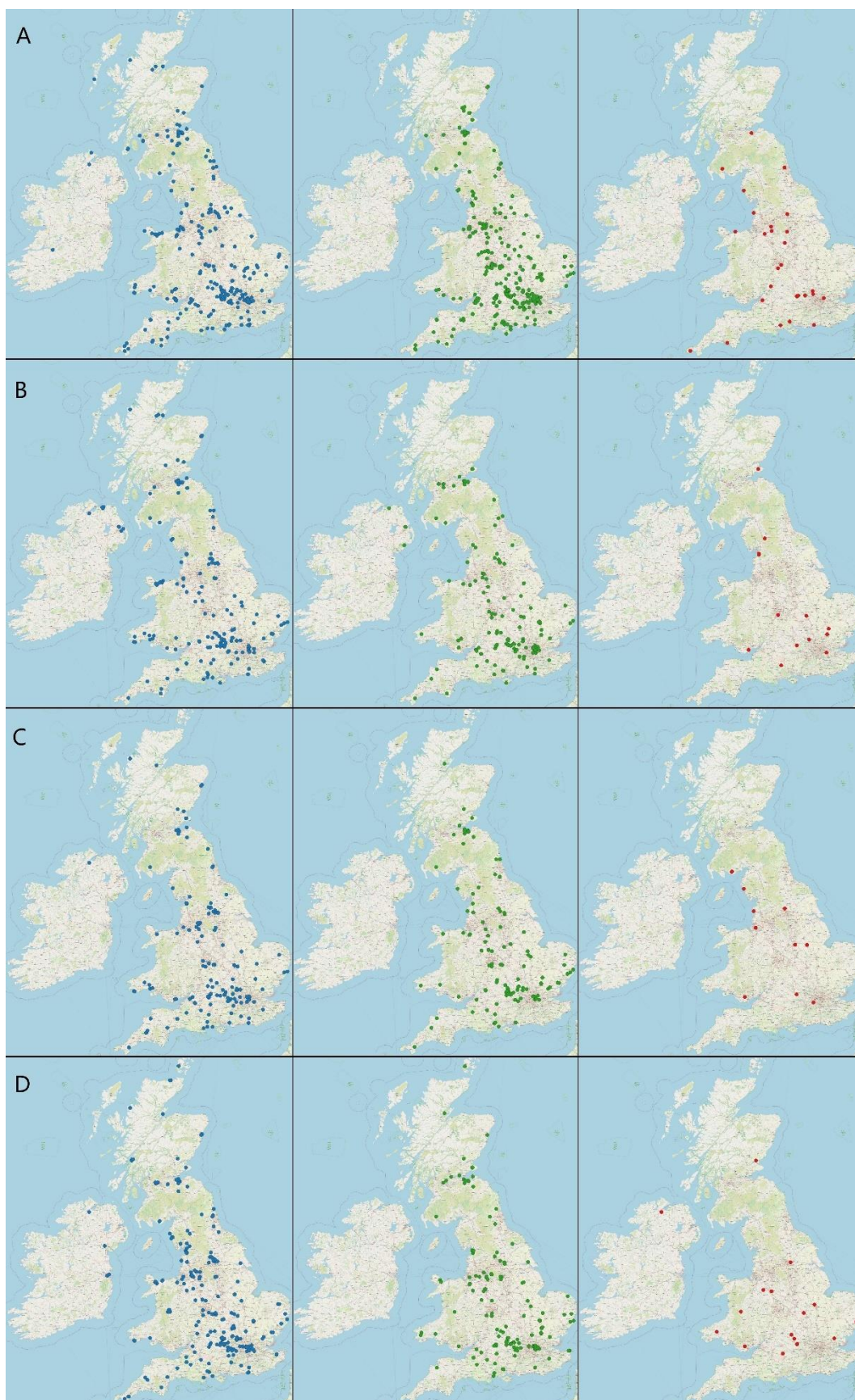


Figure 4.3: Maps showing locations of UK and Republic of Ireland air samples collected on A) 21st June 2018, B) 24th September 2018, C) 21st December 2018 and D) 20th March 2019. Blue dots indicate samples that did not grow *A. fumigatus* colonies, green dots indicate samples that did and red dots indicate samples that grew azole-resistant *A. fumigatus*.

4.3.2.1. Cross-resistance of UK tebuconazole-resistant airborne *A. fumigatus* to medical azoles

Of the 111 UK tebuconazole-resistant *A. fumigatus* isolates tested for susceptibility to the medical azoles, 85 isolates were resistant to ITZ (77%), 64 were resistant to VCZ (58%), 19 were resistant to PCZ (17%) and 83 (75%) were resistant to ISZ. Four (4%) of the tebuconazole-resistant isolates were resistant to one of the medical azoles, 19 isolates (17%) were resistant to two, 50 isolates (45%) were resistant to three and 15 isolates (14%) were resistant to all four medical azoles, while 23 isolates (21%) were fully susceptible to medical azoles. Further details of these isolates are given in Table 4.4.

Sampling date (sample prefix)	Sample (number of ARAf isolates)	County, north to south	ITZ	VCZ	PCZ	ISZ	<i>cyp51A</i> polymorphisms
21 st June 2018 (SS18)	084-02 (1)	Edinburgh	16	2	0.25	4	TR ₃₄ /L98H
	251-01 (1)	Northumberland	16	2	0.25	2	TR ₃₄ /L98H
	073-01 (0)	Dumfries & Galloway	0.25	2	0.12	0.5	
	074-02 (1)	Cumbria	16	2	0.5	2	TR ₃₄ /L98H
	068-01 (2)	Lancashire	16	2	0.25	2	TR ₃₄ /L98H
			16	2	0.25	2	TR ₃₄ /L98H
	007-02 (1)	West Yorkshire	16	2	0.25	2	TR ₃₄ /L98H
	381-02 (1)	Cheshire	16	2	0.25	2	TR ₃₄ /L98H/N479S
	202-02 (1)	South Yorkshire	16	4	0.25	2	TR ₃₄ /L98H
	403-01 (1)	Conway	16	2	0.25	2	WT
	075-01 (1)	Cheshire	16	4	0.5	4	TR ₃₄ /L98H
	257-01 (1)	Derbyshire	16	2	0.5	2	TR ₃₄ /L98H
	261-02 (2)	West Midlands	16	2	0.5	2	TR ₃₄ /L98H
			0.12	0.5	0.06	0.5	WT
	278-02 (1)	West Midlands	16	1	0.5	2	TR ₃₄ /L98H
	085-01 (1)	Worcestershire	16	2	0.25	4	TR ₃₄ /L98H
	139-01 (1)	Suffolk	16	2	0.25	4	TR ₃₄ /L98H
	177-02 (1)	Gloucestershire	16	2	0.25	2	WT
	143-01 (1)	Hertfordshire	16	2	0.25	4	TR ₃₄ /L98H
	082-01 (4)	Hertfordshire	16	4	0.5	4	TR ₃₄ /L98H
			16	4	0.5	4	TR ₃₄ /L98H
			16	2	0.25	4	TR ₃₄ /L98H
			16	2	0.25	2	TR ₃₄ /L98H
	056-02 (4)	Buckinghamshire	16	1	0.5	2	TR ₃₄ /L98H
			16	2	0.25	2	TR ₃₄ /L98H
			16	2	0.25	2	TR ₃₄ /L98H
			16	2	0.25	2	TR ₃₄ /L98H
	005-02 (1)	Oxfordshire	16	16	0.5	16	TR ₄₆ /Y121F/T289A
	013-01 (1)	Oxfordshire	16	2	0.25	2	TR ₃₄ /L98H
	040-01 (1)	Oxfordshire	16	2	0.5	2	TR ₃₄ /L98H
	375-02 (0)	Oxfordshire	0.25	1	0.06	1	

	340-01 (2)	London	16 16	2 2	0.25 0.25	2 2	TR ₃₄ TR ₃₄ /L98H	
	423-01 (1)	Bristol	0.12	0.12	0.03	0.25	WT	
	304-01 (1)	Hampshire	16	2	0.25	2	TR ₃₄ /L98H	
	193-02 (2)	West Sussex	16 0.12	1 0.25	0.5 0.03	2 0.5	TR ₃₄ /L98H WT	
	248-01 (0)	Hampshire	16	16	0.5	16		
	204-01 (3)	Devon	16 16 16	2 2 2	0.5 0.25 0.5	2 2 2	TR ₃₄ /L98H TR ₃₄ /L98H TR ₃₄ /L98H	
	285-01 (1)	Cornwall	16	2	0.25	4	WT	
24th September 2018 (AE18)	081-01 (1)	Fife	16	4	0.25	2	TR ₃₄ /L98H	
	026-01 (1)	Cumbria	16	4	0.5	4	TR ₃₄ /L98H/D481N	
	098-02 (2)	Lancashire	16 16	0.5 2	0.5 0.25	4 2	WT WT	
	098-01 (10)	Lancashire	16 16 16 16 16 16 0.25 16 16 16	2 2 2 2 2 2 1 0.25 2 2 2	0.25 0.25 0.25 0.25 0.25 0.25 0.06 0.25 0.25 0.25	4 2 2 2 2 2 2 0.25 2 2 2	D262Y WT WT WT WT WT WT TR ₃₄ /L98H WT WT WT	
	016-02 (1)	Lancashire	16	2	0.25	2	TR ₃₄ /L98H	
	060-01 (1)	West Midlands	16	2	0.25	2	TR ₃₄ /L98H	
	125-01 (1)	Northamptonshire	16	1	0.25	2	TR ₃₄ /L98H	
	124-01 (1)	Essex	16	2	0.5	2	TR ₃₄ /L98H	
	037-01 (0)	Hertfordshire	16	2	0.25	1		
	082-02 (1)	Hertfordshire	0.25	0.5	0.06	0.5	WT	
	211-01 (1)	Oxfordshire	16	1	0.25	2	TR ₃₄ /L98H	
	225-02 (0)	Cardiff	0.06	0.13	0.03	0.12		
	064-01 (1)	London	16	2	0.25	2	TR ₃₄ /L98H	
	187-01 (3)	Wiltshire	16 16 0.12	1 2 0.25	0.25 0.5 0.03	2 4 0.25	TR ₃₄ /L98H TR ₃₄ /L98H WT	
	21st December 2018 (WS18)	061-01 (0)	Dumfries & Galloway	0.12	0.12	0.03	0.25	
		061-02 (0)	Dumfries & Galloway	0.25	1	0.12	1	
		134-02 (2)	Cumbria	16 16	1 4	0.25 0.5	2 8	TR ₃₄ /L98H TR ₃₄ /L98H
060-01 (1)		West Yorkshire	ND	ND	ND	ND	TR ₃₄ /L98H	
028-02 (1)		Lancashire	16	1	0.25	2	TR ₃₄ /L98H	
153-01 (2)		Merseyside	16 16	1 1	0.25 0.25	2 2	TR ₃₄ /L98H TR ₃₄ /L98H	
119-02 (1)		Nottinghamshire	16	2	0.25	4	TR ₃₄ /L98H	
045-01 (1)		Lincolnshire	16	1	0.25	2	TR ₃₄ /L98H	
045-02 (1)		Lincolnshire	16	1	0.25	2	TR ₃₄ /L98H	
040-01 (2)		Oxfordshire	16 0.06	2 0.25	0.25 0.03	2 0.5	TR ₃₄ /L98H D262Y	
161-01 (0)		Mid Glamorgan	0.5	1	0.12	1		

20 th March 2019 (SA19)	125-01 (1)	Surrey	16	1	0.25	2	TR ₃₄ /L98H
	182-02 (1)	Dundee	0.12	0.12	0.03	0.25	WT
	178-01 (1)	Londonderry	0.06	0.12	0.03	0.25	
	122-01 (2)	West Yorkshire	16 16	1 1	0.25 0.25	1 1	
	184-01 (1)	Cheshire	0.06	0.25	0.03	0.25	S46F/R66W/ M172V/E427K
	050-01 (1)	Staffordshire	16	1	0.25	2	D262Y
	051-01 (1)	Cambridgeshire	16	1	0.25	2	WT
	170-01 (1)	Powys	16	16	0.25	16	TR ₄₆ /Y121F/T289A
	207-02 (1)	Northamptonshire	0.25	16	0.12	16	TR ₄₆ /Y121F/T289A
	176-01 (8)	Suffolk	16	1	0.25	2	WT
			16	1	0.25	2	TR ₃₄ /L98H
			16	2	0.25	2	WT
			0.06	0.25	0.03	0.25	WT
			0.12	0.25	0.03	0.5	WT
			0.06	0.25	0.03	0.25	WT
			0.06	0.25	0.03	0.25	WT
			0.06	0.25	0.03	0.25	WT
	154-02 (2)	Carmarthenshire	16 16	16 1	0.25 0.25	8 2	TR ₄₆ /Y121F/T289A TR ₃₄ /L98H
	001-03 (1)	Oxfordshire	16	1	0.25	2	TR ₃₄ /L98H
	208-01 (1)	Oxfordshire	16	2	0.25	4	TR ₃₄ /L98H/V486I
	063-01 (1)	Essex	0.12 0.5	0.25 0.5	0.03 0.03	0.25 0.5	WT
							WT
	004-01 (1)	Oxfordshire	0.25	0.25	0.03	0.5	WT
	120-01 (1)	Oxfordshire	16	2	0.25	2	TR ₃₄ /L98H
	078-02 (1)	Mid Glamorgan	16	2	0.25	2	WT
	162-01 (2)	Wiltshire	16 16	16 16	0.25 0.25	16 16	TR ₄₆ /Y121F/T289A TR ₄₆ /Y121F/T289A

Table 4.4: Details of the 111 UK tebuconazole-resistant isolates including the sampling round they were collected in, the unique identifier for the air sample they were grown from, the county from which the sample was collected, their minimum inhibitory concentrations (MICs) for itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ), and their *cyp51A* polymorphisms. Resistant MICs are highlighted in bold and isolates subsequently found not to be *A. fumigatus* are highlighted in red. WT=wild-type. ND=not done.

4.3.2.2. Confirming identity of UK airborne AR_{Af}

Several tebuconazole-resistant isolates no longer macroscopically resembled *A. fumigatus* when they were cultured for CLSI testing, or their DNA extractions failed to amplify during the PCR for the *cyp51A* promoter region, and they were subsequently determined not to be *A. fumigatus*. These isolates are highlighted in red in Table 4.4. Nine isolates, from samples SS18-073-01, SS18-248-01, SS18-375-02, WS18-061-01, WS18-061-02, WS18-161-01, SA19-063-01, SA19-122-01 and SA19-122-01, were reidentified as *A. lentulus*, which is indistinguishable from *A. fumigatus* both macro- and microscopically. Two isolates, from samples AE18-225-02 and SA19-178-01, were reidentified as *A. nidulans*, which has a deeper green coloured colony when grown on agar and can be distinguished from *A. fumigatus* under the

microscope. One isolate, from sample AE18-037-01, failed to sequence with any primers so its identity is unknown. It was decided to include these isolates in further analysis as their resistance to tebuconazole may also owe to adaptation to environmental azoles and both species are capable of causing aspergillosis.

4.3.2.3. *Cyp51A* polymorphisms in UK airborne *ARAf*

The predominant polymorphism detected in the 99 tebuconazole-resistant isolates confirmed to be *A. fumigatus* was TR₃₄/L98H ($n = 58$; 59%), with three of these isolates containing an amino acid substitution in addition to L98H. TR₄₆/Y121F/T289A was detected in six isolates (6%) and TR₃₄ without any accompanying amino acid substitutions in one isolate. 30 isolates (30%) did not contain any polymorphisms in the *cyp51A* promoter or coding regions and four isolates (4%) contained no polymorphisms in the promoter region but had amino acid substitution(s) in the coding region.

4.3.3. *ARAf* in global air samples

In the first air sampling round, 144 samples were collected globally, of which 81 (56%) samples grew 280 *A. fumigatus* isolates. In the second sampling round, 92 global samples were collected, of which 63 (68%) grew 171 *A. fumigatus* isolates. Of these *A. fumigatus* isolates, nine were resistant to tebuconazole at 6 mg/L: eight of 280 (3%) isolates from the first sampling round and one of 63 (2%) isolates from the second. Resistant isolates were collected from France ($n = 1$), Germany ($n = 2$) and the Netherlands ($n = 6$).

4.3.3.1. Cross-resistance of global tebuconazole-resistant airborne *A. fumigatus* to medical azoles

Of the nine global tebuconazole-resistant *A. fumigatus* isolates tested for susceptibility to the medical azoles, eight were resistant to ITZ, seven were resistant to VCZ, one was resistant to PCZ and eight were resistant to ISZ. Further details of these isolates are given in Table 4.5.

Sampling date (sample prefix)	Sample (number of ARAf)	Country	ITZ	VCZ	PCZ	ISZ	<i>cyp51A</i> polymorphisms
21 st June 2018 (SS18)	290-02 (1)	Germany	16	2	0.25	2	TR ₃₄ /L98H
	291-01 (1)	Germany	16	2	0.25	2	TR ₃₄ /L98H
	189-01 (3)	The Netherlands	16	2	0.25	2	TR ₃₄
			16	2	0.25	4	TR ₃₄ /L98H
			16	2	0.25	2	TR ₃₄ /L98H
	190-02 (1)	The Netherlands	16	16	0.5	16	TR ₄₆ /Y121F/T289A
	191-02 (1)	The Netherlands	16	4	0.25	2	TR ₃₄ /L98H
	192-02 (1)	The Netherlands	16	1	0.25	2	TR ₃₄ /L98H
24 th September 2018 (AE18)	235-01 (0)	France	0.12	1	0.06	0.5	

Table 4.5: Details of the nine global tebuconazole-resistant isolates including the sampling round they were collected in, the unique identifier for the air sample they were grown from, the country from which the sample was collected, their minimum inhibitory concentrations (MICs) for itraconazole (ICZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ), and their *cyp51A* polymorphisms. Resistant MICs are highlighted in bold and isolates subsequently found not to be *A. fumigatus* are highlighted in red.

4.3.3.2. Confirming identity of global airborne ARAf

One of the nine global tebuconazole-resistant isolates failed to amplify with the primers for the *cyp51A* promoter region and was reidentified as *A. lentulus*. This isolate was from sample AE18-235-01 and was collected in France.

4.3.3.3. Cyp51A polymorphisms in global airborne ARAf

The predominant *cyp51A* polymorphism detected in the eight global isolates confirmed to be *A. fumigatus* was TR₃₄/L98H (6 of 8; 75%). The isolate from the Netherlands that was pan-azole resistant contained TR₄₆/Y121F/T289A and another isolate from the Netherlands contained TR₃₄ without L98H.

4.3.4. Whole-genome sequencing of SS18 ARAf isolates

The bootstrapped phylogeny showed two large clusters of isolates and a smaller, less-distinct, cluster (Figure 4.4). The first large cluster contained 100 isolates: comprised of 12 UK SS18 isolates, five global SS18 isolates, 38 UK environmental isolates and 45 UK clinical isolates, of which 64 (64%) were azole-resistant. This cluster corresponds to previously described 'Clade A'¹⁹⁷. The second large cluster contained 102 isolates: comprised of six UK SS18 isolates, 13 UK environmental isolates and 83 UK clinical isolates, of which 26 (25%) were azole-resistant. This cluster corresponds to previously described 'Clade B'¹⁹⁷. The smaller cluster contained 55 isolates: comprised of 19 UK SS18 isolates, three global SS18 isolates, 14 UK environmental

isolates and 19 UK clinical isolates, of which 52 (95%) were azole-resistant. The 22 SS18 isolates all contain TR₃₄/L98H; therefore this cluster corresponds to previously described 'Clade Aa'²³¹.

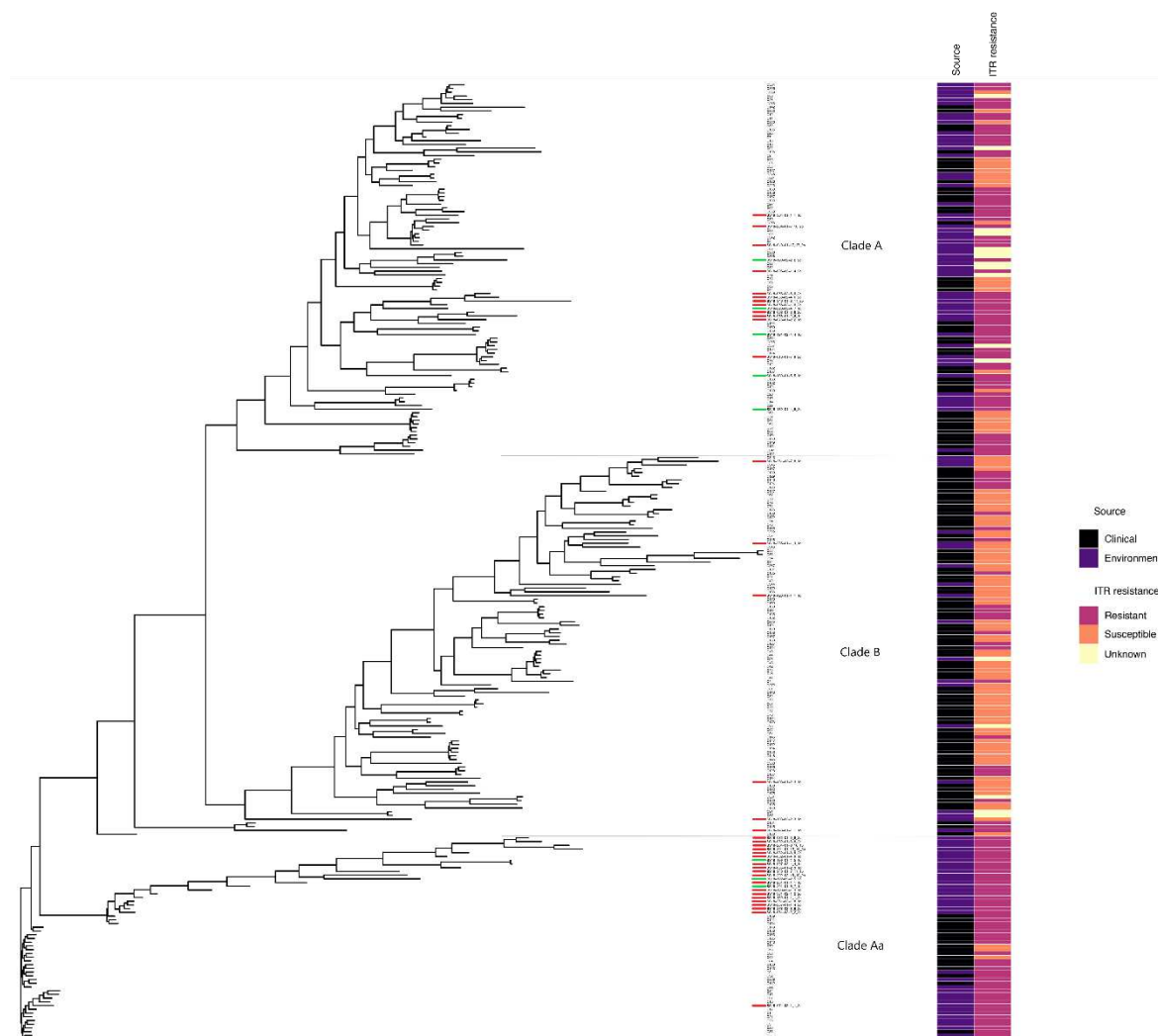


Figure 4.4: Bootstrapped phylogeny of 212 clinical and environmental *A. fumigatus* isolates collected from across the UK between 2005 and 2017²³¹, 37 tebuconazole-resistant *A. fumigatus* isolates collected from the UK in the first air sampling round (prefix SS18) and eight tebuconazole-resistant *A. fumigatus* isolates collected globally in the first air sampling round (prefix SS18). UK SS18 isolates are denoted by red lines and global SS18 isolates by green lines.

4.3.5. Maximum air temperatures on air sampling dates

Of the 1,894 samples collected across the four sampling rounds, 1,503 were collected in England, 237 in Scotland, 128 in Wales, 22 in Northern Ireland (NI) and four in Republic of Ireland. As the Met Office data was only available for the UK, the four samples from the Republic of Ireland were dropped from analyses involving maximum air temperature (two samples from the first air sampling and two samples from the fourth sampling round). In the

first sampling round (summer), maximum air temperature ranged from 14.0-27.2°C and samples were collected on six separate days. In the second sampling round (autumn), maximum air temperature ranged from 12.5-23.7°C and samples were collected on 12 days. In the third round (winter), maximum temperatures ranged from 2.7-13.5°C between 16 sampling days and in the fourth round (spring), temperatures ranged from 8.5-18.8°C between 14 sampling days. The range of maximum daily temperatures for each sampling round are shown in Figure 4.5 and a breakdown of maximum air temperatures by dates on which samples were collected is shown in Appendix 13.

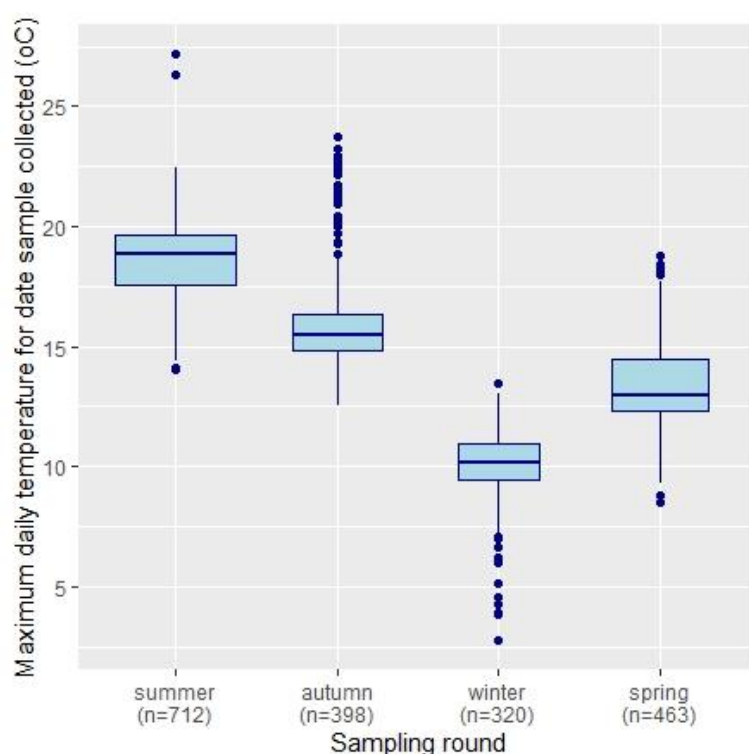


Figure 4.5: Boxplot showing the maximum daily temperatures at the locations that samples were collected from for each sampling round.

4.3.6. Land cover classifications of UK sampling locations

There were 803 unique locations in the UK and Republic of Ireland that air samples were collected from across the four sampling rounds: 630 locations in England, 96 in Scotland, 59 in Wales, 15 in NI and three in the Republic of Ireland (Figure 4.6). As there was no LCM available for the Republic of Ireland these three locations were dropped from analyses involving land cover classification. Between GB and NI, 64 (8%) locations were rural; classified by the LCM as 1 or 2 (woodland), 3 (arable) and 4, 5 or 10 (grassland), and 736 (92%) were

urban; classified as 20 (urban) or 21 (suburban). The breakdown of land cover classifications for sampling locations by country is shown in Table 4.6.

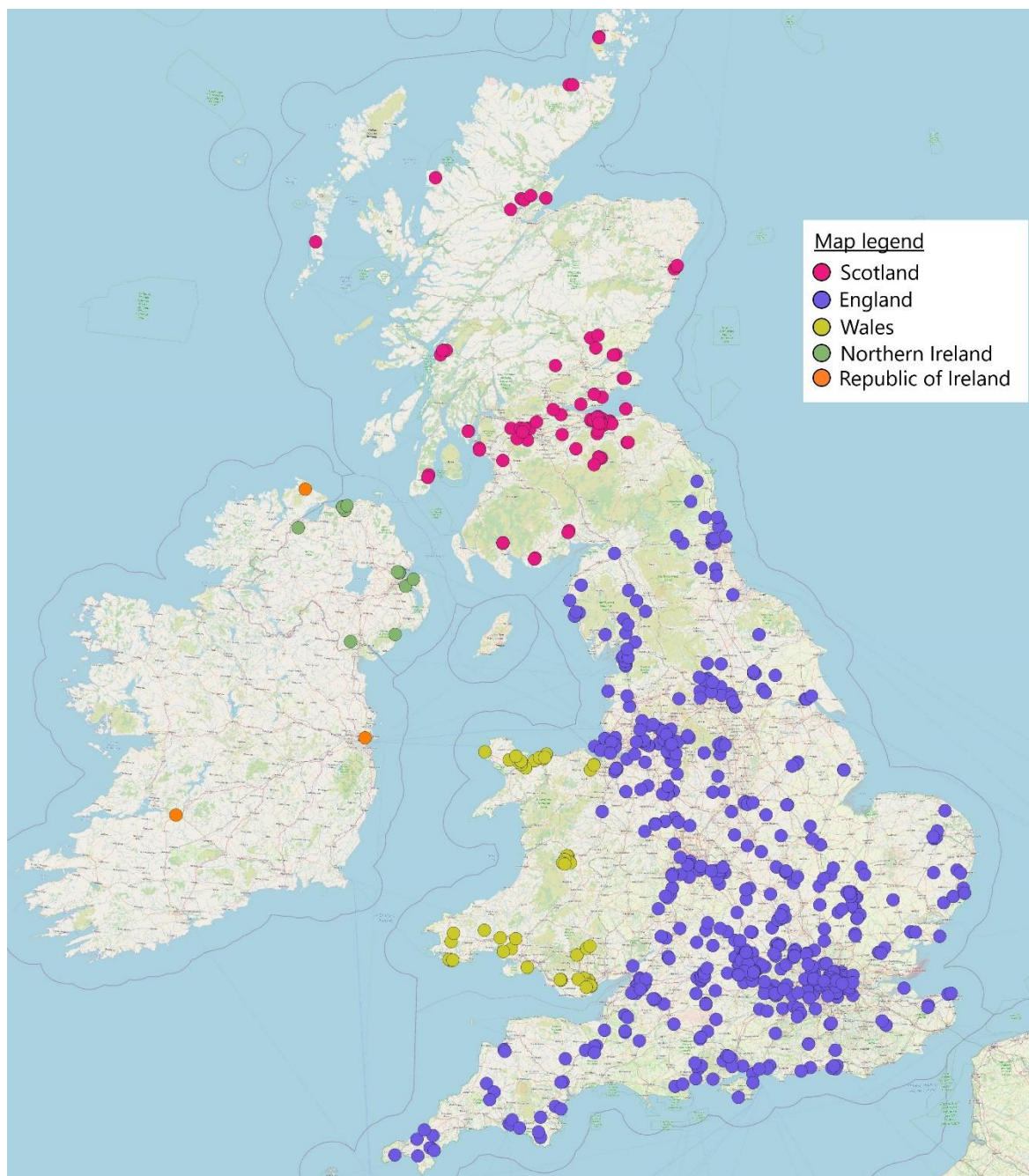


Figure 4.6: The 803 unique locations in the UK and Republic of Ireland that air samples were collected from across the four sampling rounds, coloured by country.

Land cover classification	Sampling locations				Totals
	England	Scotland	Wales	Northern Ireland	
1 – Deciduous woodland	5	5	0	0	10
2 – Coniferous woodland	0	1	0	0	1
3 – Arable	7	1	1	0	9
4 – Improved grassland	22	7	11	1	41
5 – Neutral Grassland	2	0	0	0	2
10 – Heather grassland	1	0	0	0	1
Rural total (% of locations):	37 (6)	14 (15)	12 (20)	1 (7)	64 (8)
20 – Urban	188	24	6	4	222
21 – Suburban	405	58	41	10	514
Urban total (% of locations):	593 (94)	82 (85)	47 (80)	14 (93)	736 (92)
Total:	630	96	59	15	800

Table 4.6: Breakdown of land cover classes for air sampling locations by country, obtained from UK Centre for Ecology & Hydrology’s 2019 land cover maps for Great Britain and Northern Ireland.

4.3.7. Land cover classifications of composting facilities with open windrow or outdoor activity in the UK

The land cover classifications were also obtained for the 257 OW/OA composting facilities in the UK, to ensure that any association between the proximity of a sampling location to an OW/OA composter and its likelihood of growing *A. fumigatus* was not confounded by the composter’s location. Across the UK, the locations of OW/OA composting facilities were approximately evenly split between rural (53%) and urban (47%) locations. The breakdown of land cover classifications of OW/OA composting facilities by country is shown in Table 4.7.

Land cover classification	Composting facilities with open windrow or outdoor activity			
	England	Scotland	Wales	Northern Ireland
1 – Deciduous woodland	10	0	2	0
2 – Coniferous woodland	0	0	1	0
3 – Arable	31	4	2	2
4 – Improved grassland	55	3	10	3
5 – Neutral grassland	3	0	0	2
7 – Acid grassland	1	0	1	0
12 – Inland rock	0	0	2	1
16 – Supralittoral sediment	0	1	0	0
19 – Saltmarsh	2	0	0	0
Rural total (% of locations):	102 (49)	8 (57)	18 (67)	8 (100)
20 – Urban	27	3	6	0
21 – Suburban	79	3	3	0
Urban total (% of locations):	106 (51)	6 (43)	9 (33)	0 (0)
Total:	208	14	27	8

Table 4.7: Breakdown of land cover classifications for locations of composting facilities in the UK with open windrow or outdoor activity by country. Land cover data were obtained from UK Centre for Ecology & Hydrology's 2019 land cover maps for Great Britain and Northern Ireland.

4.3.8. Distances from sampling locations to nearest composting facility

The 800 unique sampling locations across England, Scotland, Wales and NI were used to calculate distance to the nearest OW/OA composting facility. The closest distance between a sampling location and composting site was 0 km, as a composting site constituted the workplace of a citizen science participant, and the furthest distance was 148 km. In total, 32 sampling locations were within 2 km of the nearest composting facility. Figure 4.7A shows the distances between sampling locations and the closest OW/OA facility.

All current UK postcodes ($n = 1,773,498$) were downloaded and the distance between them and the nearest OW/OA facility calculated. There were 11 postcodes that were 0 km from a OW/OA composting facility and 47,315 postcodes within 2 km. The furthest distance between a postcode and nearest composter was 367 km. Figure 4.7B shows the distances between UK postcodes and the nearest OW/OA composting facility for the 1,771,918 postcodes that were within 150 km of the nearest composter, to use the same distance scale as Figure 4.7A.

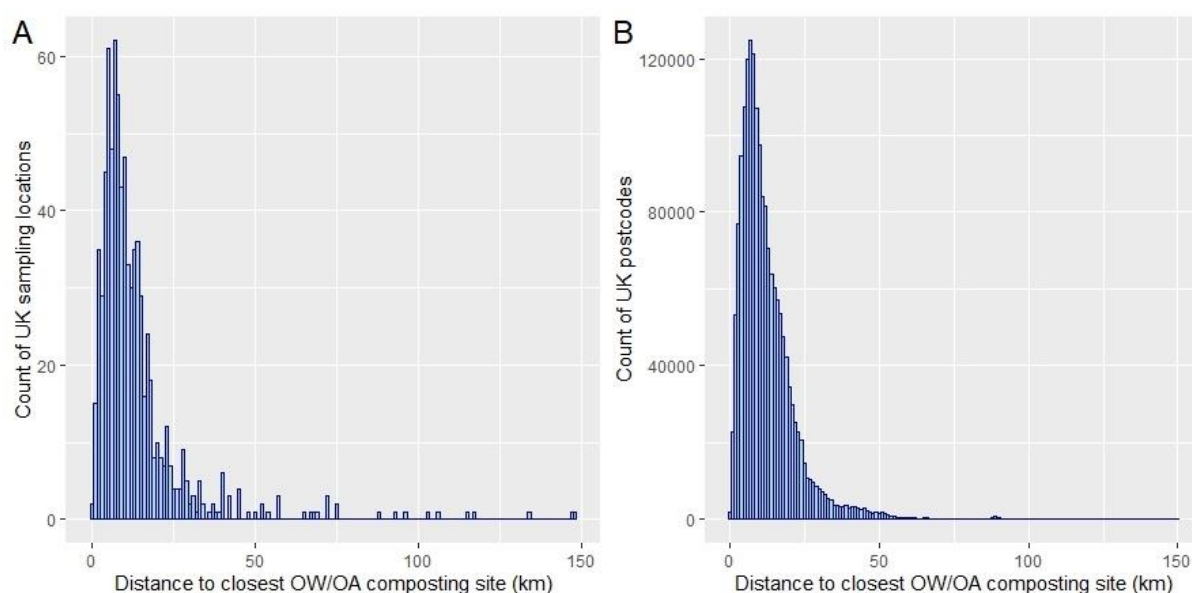


Figure 4.7: Histogram showing distances between A) UK air sampling locations ($n = 800$) and B) all UK postcodes ($n = 1,771,918$) and the closest composting facility with open windrow or outdoor activity within 150 km.

4.3.9. Percentage of arable land in 2 km buffer surrounding sampling locations

The percentage of arable land (land cover class 3) in a 2 km buffer around sampling location was calculated for 800 unique locations in England, Wales, Scotland and NI. Of these locations, 115 (14%) did not have any arable land within 2 km and the remainder had between 0.0003% and 81%. As shown in Table 4.8, Wales had the highest proportion (39%) of sampling locations without arable land in the surrounding 2 km buffer and NI had no sampling locations without arable land in their surroundings.

Country	Number of unique sampling locations	Samples without arable land in 2 km buffer (% of samples)	Percentage of arable land in 2 km buffer of remaining sampling locations (%)
England	630	68 (11)	0.0003-81
Scotland	96	24 (25)	0.05-78
Wales	59	23 (39)	0.04-26
Northern Ireland	15	0 (0)	2-43

Table 4.8: Breakdown by country of the percentage of arable land within surrounding 2 km of unique sampling locations.

4.3.10. Factors influencing whether samples grew *A. fumigatus*

4.3.10.1. Logistic regression model for UK samples

The logistic regression on UK samples ($n = 1,889$) to determine which factors influenced whether samples grew *A. fumigatus* excluded Republic of Ireland samples ($n = 4$), because data for several independent variables were not available, and one UK sample, because the incorrect postcode was provided. The independent variables that were, individually, found to have a significant effect on whether a sample grew *A. fumigatus* were: sampling round ($\chi^2 = 50.3$, $df = 3$, $p < 0.01$), date that sample was collected ($\chi^2 = 132.5$, $df = 51$, $p < 0.01$), maximum daily temperature ($\chi^2 = 29.3$, $df = 1$, $p < 0.01$), land cover classification of sampling location ($\chi^2 = 17.4$, $df = 7$, $p = 0.01$), proximity to nearest OW/OA composting facility ($\chi^2 = 15.1$, $df = 1$, $p < 0.01$) and land cover classification of nearest OW/OA composting facility ($\chi^2 = 26.5$, $df = 9$, $p < 0.01$). Percentage coverage of arable land in the 2 km surrounding sampling location had a borderline significant effect on whether a sample grew *A. fumigatus* ($\chi^2 = 3.7$, $df = 1$, $p = 0.05$). The relationships between each significant independent variable and the growth of *A. fumigatus* from samples are shown in Figure 4.8.

The model that gave the lowest AIC included sampling round, maximum daily temperature at sampling location, land cover classification of sampling locations, percentage of arable land in 2 km buffer around sampling location, proximity of sampling location to nearest OW/OA composting facility and land cover classification of nearest OW/OA composting facility. The odd ratios and p-values for these independent variables when analysed together are shown in Table 4.9. Samples collected in autumn and spring had significantly lower odds of growing *A. fumigatus* relative to samples collected in the summer ($p = 0.03$ and $p < 0.01$, respectively). There was no significant difference in the odds of growing *A. fumigatus* from samples collected in winter, compared to samples collected in summer. In this model, maximum daily temperature at sampling location no longer had a significant effect on whether a sample grew *A. fumigatus*. Sampling locations with land cover classified as 4 (improved grassland) had lower odds of growing *A. fumigatus* than sampling locations with land cover classified as 1 (deciduous woodland) that was marginally significant ($p = 0.05$) but no other significant associations were observed for land cover of sampling location. Increased percentage coverage of arable land in the 2 km surrounding sampling location slightly, yet significantly ($p = 0.03$), increased the odds of a sample growing *A. fumigatus*. Increasing distance between

sampling location and the nearest OW/OA composting facility slightly, yet significantly ($p = 0.02$), decreased the odds of a sample growing *A. fumigatus*. Sampling locations nearest to OW/OA composting facilities with land cover classified as 7 (acid grassland) and 12 (inland rock) had significantly lower odds ($p = 0.03$ and $p = 0.05$, respectively) of growing *A. fumigatus* compared to those closest to facilities with land cover classified as 1 (deciduous woodland).

Independent variable	Odds ratio (95% CI)	Pr(> z)
Sampling round		
<i>summer (baseline)</i>		
<i>autumn</i>	0.73 (0.55-0.96)	0.03
<i>winter</i>	0.82 (0.48-1.39)	0.47
<i>spring</i>	0.49 (0.34-0.71)	<0.01
Maximum daily temperature at sampling location on sampling date	1.03 (0.98-1.08)	0.29
Land cover classification of sampling location		
1 (baseline) – Deciduous woodland		
2 – Coniferous woodland	- ^a	0.98
3 – Arable	0.36 (0.11-1.10)	0.08
4 – Improved grassland	0.38 (0.14-0.97)	0.05
5 – Neutral grassland	0.71 (0.03-19.81)	0.82
10 – Heather grassland	- ^a	0.97
20 – Urban	0.55 (0.22-1.27)	0.17
21 – Suburban	0.45 (0.19-1.02)	0.06
Percentage arable land in 2 km buffer surrounding sampling location	1.01 (1.00-1.01)	0.04
Proximity of sampling location to nearest OW/OA composting facility	0.99 (0.99-0.99)	<0.01
Land cover classification of nearest OW/OA composting facility		
1 (baseline) – Deciduous woodland		
3 – Arable	0.72 (0.39-1.29)	0.26
4 – Improved grassland	0.60 (0.33-1.08)	0.09
5 – Neutral grassland	0.93 (0.44-1.98)	0.85
7 – Acid grassland	0.18 (0.03-0.75)	0.03
12 – Inland rock	0.12 (0.01-0.78)	0.05
16 – Supralittoral rock	1.24 (0.46-3.38)	0.67
19 – Saltmarsh	- ^a	0.97
20 – Urban	0.79 (0.43-1.42)	0.43
21 – Suburban	0.81 (0.45-1.44)	0.47

Table 4.9: Odds ratios, confidence intervals and p-values calculated for independent variables included in a logistic regression model using samples collected in the UK ($n = 1,889$) to explain whether a sample grew *A. fumigatus*. Significant results ($p \leq 0.05$) are highlighted in bold.

^aInsufficient data to calculate odds ratio and confidence intervals.

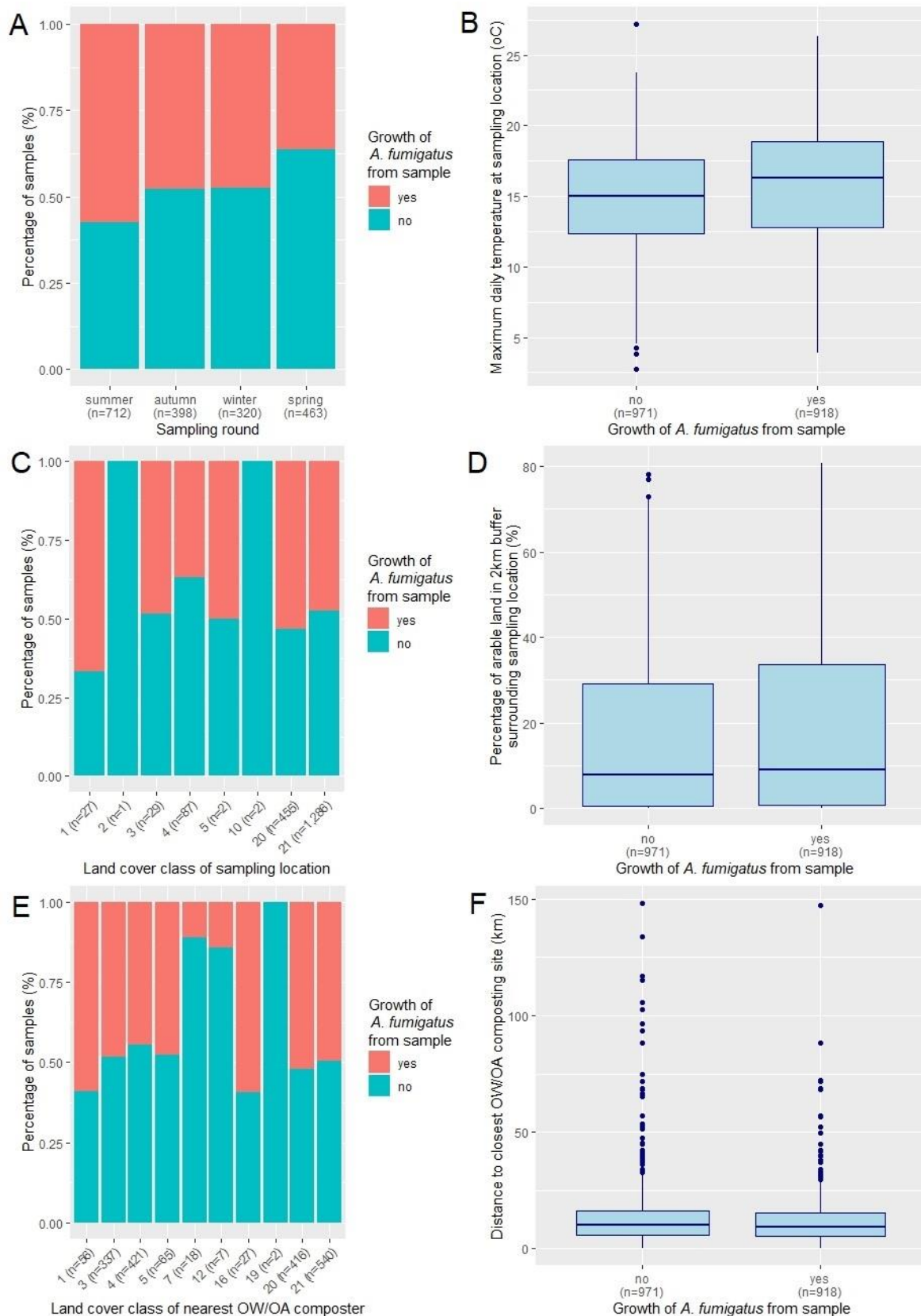


Figure 4.8: Plots showing the relationship between whether a sample grew *A. fumigatus* and A) sampling round that sample was collected in, B) maximum daily temperature at sampling location on sampling date, C) land cover classification of sampling location, D) percentage of arable land in the 2 km buffer surrounding sampling location, E) land cover classification of nearest composting facility with open windrow or outdoor activity (OW/OA), and F) proximity of sampling location to nearest OW/OA composting facility.

4.3.10.2. Geographical weighted regression models for UK samples

Geographical weighted regressions (GWRs) were run separately for UK samples collected in summer ($n = 710$), autumn ($n = 398$), winter ($n = 320$) and spring ($n = 461$), to account for the significant effect of sampling round on the likelihood of a sample growing *A. fumigatus* seen in the logistic regression. Only continuous independent variables that were previously found to be significantly associated with the outcome were included in the GWRs. These were: ‘maximum daily temperature at sampling location on sampling date’, ‘percentage of arable land in the 2 km buffer surrounding sampling location’ and ‘proximity of sampling location to the nearest OW/OA composting facility’.

All four GWRs found that accounting for sampling location increased the percentage of deviance explained by the model, compared to the GLMs that did account for spatial variation (Table 4.10). The greatest increase in deviance explained was for samples collected in summer, from 0.7% in the GLM to 6.1% in the GWR, and the least improvement was seen for samples collected in winter, from 2.0% to 3.6%.

	Input data for GWR			
	Samples collected in summer ($n = 710$)	Samples collected in autumn ($n = 398$)	Samples collected in winter ($n = 320$)	Samples collected in spring ($n = 461$)
Deviance explained by GLM ^a (%)	0.7	2.6	2.0	1.8
Deviance explained by GWR (%)	6.1	4.9	3.6	6.1
Maximum daily temperature				
<i>number of significant associations</i>	43	0	64	6
<i>significant negative associations</i>	0	0	0	6
<i>significant positive associations</i>	43	0	64	0
<i>range of significant odds ratios</i>	1.28-1.56	NA	1.19-1.26	0.75-0.77
Arable land percentage in 2 km buffer				
<i>number of significant associations</i>	118	207	60	120
<i>significant negative associations</i>	116	207	0	0
<i>significant positive associations</i>	2	0	60	120
<i>range of significant odds ratios</i>	0.98-1.03	0.99	1.01-1.02	1.01-1.03
Proximity to nearest OW/OA composter				
<i>number of significant associations</i>	14	134	0	0
<i>significant negative associations</i>	14	134	0	0
<i>significant positive associations</i>	0	0	0	0
<i>range of significant odds ratios</i>	0.99	0.99	NA	NA

Table 4.10: Summary of geographically weighted regression outputs for UK samples, run separately for each sampling round. ^aGLM refers to the non-spatial logistic regression model with growth of *A. fumigatus* as the binary outcome.

Allowing the relationship between dependent and independent variables to vary by location allowed considerable variation across the UK, which can be visualised for maximum daily temperature in Figure 4.9 where positive associations between increasing temperature and likelihood of growing *A. fumigatus* are shown in red and negative associations are shown in blue for the four sampling rounds.

The relationship between maximum daily temperature at sampling location on sampling date and likelihood of growing *A. fumigatus* changed with season: in summer 43 of 43 (100%) and winter 64 of 64 (100%) of statistically-significant associations were positive, whereas in spring six of six (100%) of statistically-significant associations were negative. Positive associations indicate that samples grew more *A. fumigatus* colonies when the maximum daily temperature was higher, while the opposite is true for negative associations. None of the associations for maximum daily temperature were significant in autumn.

The relationship between increasing percentage of arable land in surrounding 2 km buffer and likelihood of growing *A. fumigatus* also changed with season: in summer 116 of 118 (98%) and autumn 207 of 207 (100%) of statistically-significant associations were negative, whereas in winter 60 of 60 (100%) and spring 120 of 120 (100%) were positive. The size of the odds ratios suggests the associations for maximum daily temperature are stronger than those for percentage of arable land in 2 km buffer surrounding sampling location, which are marginally significant.

The relationship between proximity of sampling location to the nearest OW/OA composter and likelihood of growing *A. fumigatus* also depended on season: in summer 14 of 14 (100%) and autumn 134 of 134 (100%) of statistically-significant associations were negative, whereas in winter and spring none of the associations were significant. Negative associations indicate that sampling locations with greater distance between them and the nearest OW/OA composter grew fewer *A. fumigatus* colonies.

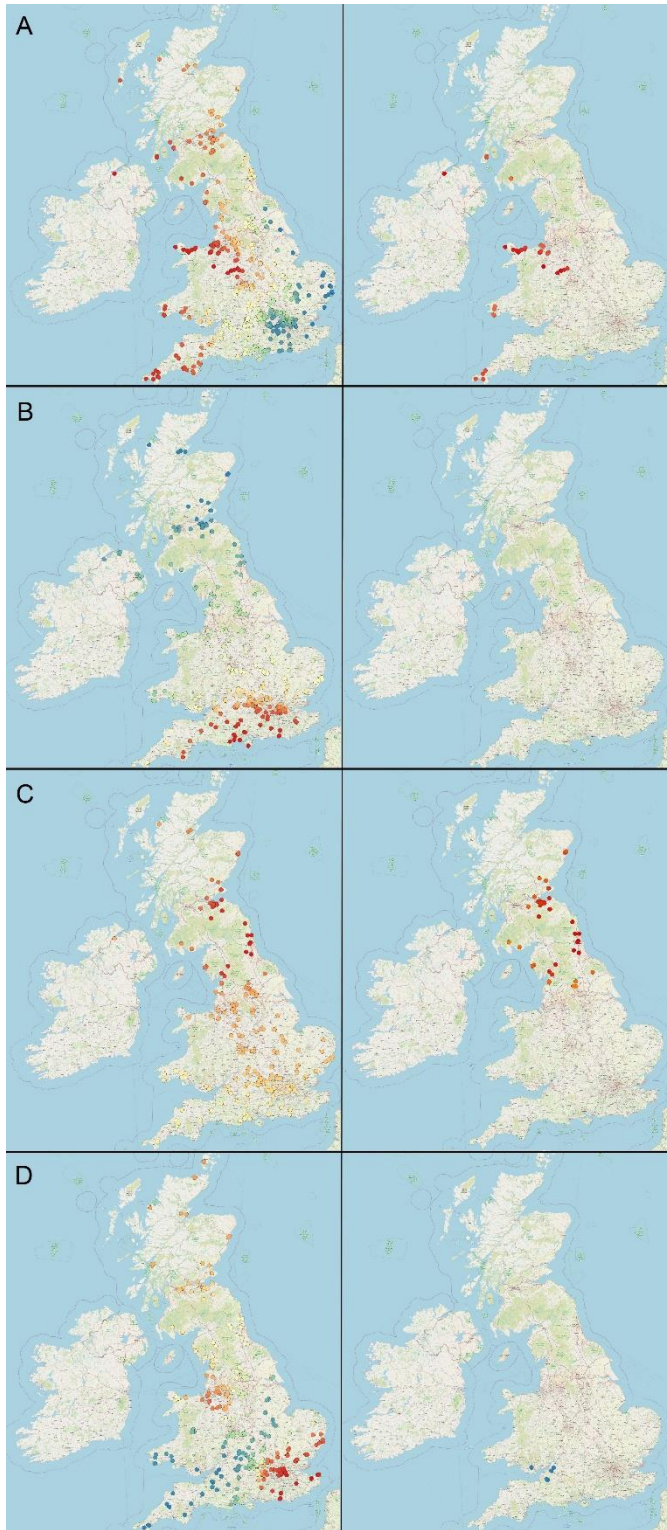


Figure 4.9: Geographically weighted regression coefficients for the relationship between increasing maximum daily temperature and likelihood of growing *A. fumigatus* at each sampling location for samples collected in A) summer, B) autumn, C) winter, and D) spring. All coefficients are shown on the left and coefficients for which the association was significant ($p \leq 0.05$) are shown on the right. Positive associations are shown in red and negative associations are shown in blue, with darker colours indicating stronger associations.

4.3.11. Factors influencing the number of *A. fumigatus* colonies grown from a sample

4.3.11.1. Negative binomial model for UK samples

In the negative binomial regression model run on UK samples that grew *A. fumigatus* ($n = 918$) the independent variables that were, individually, found to have a significant effect on number of *A. fumigatus* colonies grown were: sampling round ($\chi^2 = 35.9$, $df = 3$, $p < 0.01$), date sample was collected ($\chi^2 = 76.2$, $df = 36$, $p < 0.01$) and proximity of sampling location to the nearest OW/OA composting facility ($\chi^2 = 10.2$, $df = 1$, $p < 0.01$). Whether the nearest OW/OA composting facility was in an urban or rural location had a marginally significant effect ($\chi^2 = 3.8$, $df = 2$, $p = 0.05$). The relationships between each significant independent variable and the number of *A. fumigatus* colonies grown from samples are shown in Figure 4.10.

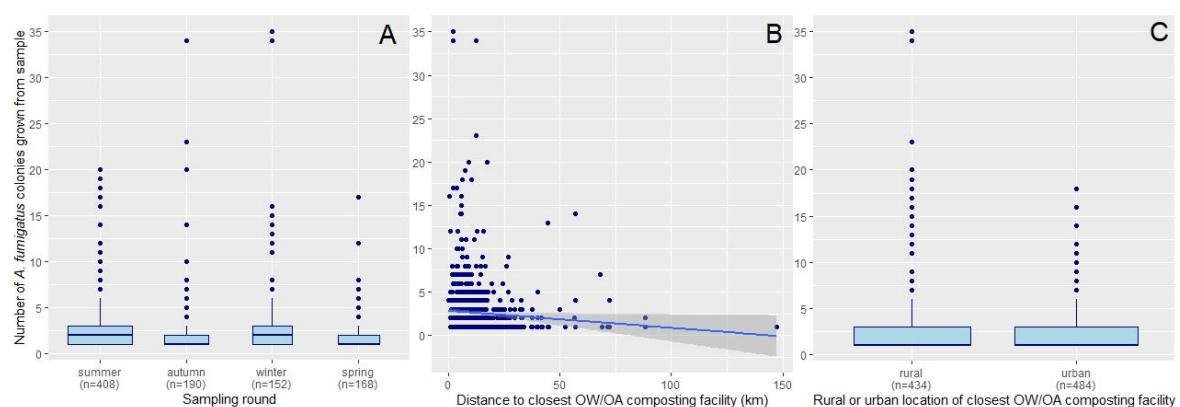


Figure 4.10: Plots showing the relationship between the number of *A. fumigatus* colonies grown from samples and A) sampling round that sample was collected in, B) proximity of sampling location to nearest composting facility with open windrow or outdoor activity (OW/OA), and C) rural or urban location of nearest OW/OA composting facility to sampling location.

The model with lowest AIC included sampling round, proximity of sampling location to the nearest OW/OA composting facility and whether the nearest OW/OA composting facility was in an urban or rural location. Samples collected in autumn and spring grew significantly reduced numbers of *A. fumigatus* colonies compared to samples collected in summer (both $p < 0.01$). There was no significant difference in numbers of colonies grown from samples collected in winter compared to those collected in summer. Increasing distance of sampling location from the nearest OW/OA composting facility was significantly associated with a reduction in the number of *A. fumigatus* colonies grown from samples ($p < 0.01$). Samples with locations closest to urban OW/OA composting facilities (landcover classifications 20 or

21) grew significantly reduced numbers of *A. fumigatus* colonies compared to samples with locations closest to rural OW/OA composting facilities ($p = 0.04$).

4.3.12. Factors influencing whether samples grew ARAf

4.3.12.1. Logistic regression model for UK samples

All UK samples except those collected from the Republic of Ireland and the incorrect postcode ($n = 1,889$) were included in a logistic regression model to determine which factors influenced whether a sample grew ARAf. None of the independent variables tested significantly improved upon the null model in explaining the outcome.

4.3.13. Factors influencing the number of ARAf colonies grown from a sample

4.3.13.1. Negative binomial model for UK samples

UK samples that grew ARAf ($n = 73$) were included in a negative binomial regression model to determine which factors influenced the number of ARAf colonies grown. None of the independent variables tested significantly improved upon the null model in explaining the outcome.

4.4. Discussion

The primary aim of this chapter was to ascertain baseline concentrations of airborne *A. fumigatus* and ARAf spores in the UK. To do this, it was important to collect a large number of air samples in a standardised manner with a wide geographical distribution that did not focus on specific locations such as hospitals or composting facilities. The citizen science approach used in this thesis meant that the 1,894 air samples collected far exceeded the number collected by existing studies; such as 144 air samples collected by Tsitsopoulou *et al.* (2018)¹³⁴ and 150 air samples collected by Bromley *et al.* (2014)¹³². Furthermore, samples were collected from all over England, Wales, Scotland and NI meaning they had greater geographical coverage than existing studies that took place in Cardiff⁶⁶, South Wales¹³⁴, Manchester^{65,132}, Dublin¹⁹⁰ and Harpenden⁸⁹. Sample collection was standardised by sending participants two identical air samplers and asking them to expose them for 6-10 hours on the same day, or as close to the sampling date as possible. While the use of passive air samplers meant that spore concentrations could not be converted into CFUs/m³ as for active air

samplers, it was possible to report the proportion of samples that grew *A. fumigatus*/ARAf and the numbers of *A. fumigatus*/ARAf colonies grown per sample. Overall, 919 of 1,894 (49%) samples grew *A. fumigatus* colonies and 73 samples (8%) grew ARAf colonies. A total of 2,366 *A. fumigatus* isolates were cultured from 919 samples, which averaged 2.6 colonies per sample, and 111 (5%) isolates grew in the presence of tebuconazole at 6 mg/L. 12 of these isolates were subsequently found not to be *A. fumigatus*, with nine re-identified as *A. lentulus*, two re-identified as *A. nidulans* and one unknown, but they were included in subsequent analyses because of their acquired resistance to one or more azoles and their potential to cause aspergillosis^{233,234}. The comparability of results between different sampling locations and rounds allowed analyses to detect associations between environmental factors and the likelihood of growing *A. fumigatus*/ARAf or the numbers of *A. fumigatus*/ARAf colonies grown.

The secondary aim of this chapter was to establish whether any airborne ARAf spores circulating in the UK were resistant to medical azoles, as well as to the agricultural azole tebuconazole. Of the 111 tebuconazole-resistant isolates, the majority were also resistant to ITZ (77%), VCZ (58%) or ISZ (75%), whereas fewer were cross-resistant to PCZ (17%). 23 isolates (21%) were not resistant to any of the medical azoles, whereas four isolates (4%) were resistant to one, 19 isolates (17%) were resistant to two, 50 isolates (45%) were resistant to three and 15 isolates (14%) were pan-azole resistant. The implications of these findings for human health are that 1 in 20 of the spores *A. fumigatus* spores we inhale daily have acquired some form of azole-resistance, 1 in 25 spores are resistant to at least one medical azole, and 1 in 140 spores are pan-azole resistant. As it is estimated that all humans inhale several hundred *A. fumigatus* spores every day⁵¹, it is highly likely that everyone in the UK is inhaling several ARAf spores every day.

The tertiary aim of this chapter was to characterise the polymorphisms responsible for azole-resistance in UK isolates, which was done by sequencing the promoter and coding regions of the *cyp51A* gene. Similar to Dunne *et al.* (2017)¹⁹⁰ and Fraaije *et al.* (2020)⁸⁹, the most common *cyp51A* polymorphism identified was TR₃₄/L98H, which was detected in 58 isolates; three of which contained an amino acid substitution in addition to L98H. Isolates with TR₃₄/L98H typically had MICs to ITZ of 16 mg/L, 2 or 4 mg/L to VCZ and ISZ, and 0.25 or 0.5 mg/L to PCZ, meaning they were resistant to three or four of the medical azoles. One isolate

contained TR₃₄ without any accompanying amino acid substitutions, yet had the same resistance profile to medical azoles as isolates with TR₃₄/L98H. TR₄₆/Y121F/T289A was detected in six isolates; the majority of which had the highest MIC of 16 mg/L to ITZ, VCZ and ISZ, but were susceptible to PCZ. Four isolates had no tandem repeat but had a single amino acid substitution in *cyp51A* and the MICs to medical azoles were highly variable between these isolates. Several isolates ($n = 30$) did not contain any polymorphisms in *cyp51A*, despite being resistant to one or more medical azole in addition to tebuconazole. The azole-resistance in these isolates could be a result of polymorphisms in non-*cyp51A* genes^{235–237}, mechanisms such as efflux pumps^{235,236} or cholesterol import²³⁵, or transcriptional activity in conidia in response to environmental stresses²³⁸.

While the focus of this thesis was to collect *A. fumigatus* from across the UK, the global nature of Twitter meant that samples were also sent from other countries. Across the summer and autumn sampling rounds, 236 global samples were collected that grew 451 *A. fumigatus* isolates of which nine (2%) were resistant to tebuconazole at 6 mg/L. These nine isolates were collected from France, Germany and the Netherlands and were cross-resistant to ITZ ($n = 8$), VCZ ($n = 7$) and ISZ ($n = 8$). Only one isolate was also resistant to PCZ, making it pan-azole resistant, and this was found to contain TR₄₆/Y121F/T289A. Similar to the UK samples, the predominant *cyp51A* polymorphism identified in these isolates was TR₃₄/L98H and one isolate had no polymorphisms. The isolate from France was re-identified as *A. lentulus*.

The final aim of this chapter was to determine whether environmental variables influenced a) whether a sample was likely to grow *A. fumigatus* or ARAf, or b) the number of *A. fumigatus* or ARAf spores grown from a sample. The strongest predictor of whether a sample grew *A. fumigatus* and the number of *A. fumigatus* colonies grown per sample was the sampling round in which a sample was collected. Samples collected in the autumn and spring sampling rounds were significantly less likely to grow *A. fumigatus* and grew significantly fewer *A. fumigatus* colonies than samples collected in summer, whereas no significant differences were found for samples grown in winter. These findings differ to existing studies looking at seasonal variation in *A. fumigatus* spore concentrations. Alshareef and Robson (2014)⁶⁵ did not find any season variation in spore concentrations during their 2-year study sampling air in Manchester, while both MAARA in Derby and the National Pollen and Aerobiology Research Unit at the University of Worcester found *Aspergillus*/*Penicillium* spore concentrations to be

highest in autumn. It is unlikely that the passive air samplers used in this thesis, as opposed to the active air samplers used by these studies, would account for these differences. However, it is possible that the effect of season could vary by the geographical location of sampling, as seen in this study for the variables included in the GWRs, such that sampling in a fixed location may not capture seasonal effects elsewhere. It is also possible that the MAARA and National Pollen and Aerobiology Research Unit counts were inflated by the inclusion of *Penicillium* spores.

Additional environmental variables that were found to have a statistically-significant effects on whether a sample grew *A. fumigatus* were land cover classification of sampling location, the percentage of arable land in the 2 km buffer surrounding sampling location, the proximity of the sampling location to the nearest OW/OA composter, and the land cover classification of this composter. Samples collected from locations classified as improved grassland ($n = 87$) were significantly less likely to grow *A. fumigatus* than samples collected from deciduous woodland ($n = 27$). This may be due to air currents flowing more freely in grassland than woodland making spores more likely to disperse than settle on the air samples, or due to greater growth of *A. fumigatus* on fallen leaves and damp bark in woodland. An increasing percentage of arable land in the 2 km surrounding sampling location was associated with an increased likelihood of the sample growing *A. fumigatus*, which might be explained by farmers spreading compost or manure on their fields preceding growing season or by decomposing crop stubble post-growth. An increasing distance between sampling location and the nearest OW/OA composter was associated with a decreased likelihood of growing *A. fumigatus*, which is to be expected with the drop-off in *A. fumigatus* spore concentrations with distance from composter demonstrated by existing studies^{61–63,187,239}.

Allowing the effect of environmental variables to vary according to sampling location in the GWR explained a greater percentage of the variance in the data than by not accounting for location in the GLM. This was because at some sampling locations there was a positive association between increasing maximum daily temperature, increasing percentage of arable land in surrounding 2 km and increasing distance from nearest OW/OA composter and likelihood of growing *A. fumigatus*, while at other sampling locations these associations were negative. However; the associations that were statistically-significant were predominantly in the same direction. As a thermotolerant fungus, it would make sense for increasing

temperatures to be beneficial for *A. fumigatus* growth and to reduce competition from other environmental microbes. Similarly, increasing percentage of arable land in the 2 km buffer surrounding sampling location significantly increased the odds of growing *A. fumigatus* for samples collected in winter and spring, but decreased the odds for samples collected in summer and autumn. These variations may well be due to farming practices on the arable land: spreading of compost or manure at different times might be a source of *A. fumigatus* spores, whereas applications of fungicides should result in an overall reduction in *A. fumigatus* spores.

Environmental variables that influenced the number of *A. fumigatus* spores grown from a sample, in addition to sampling round, were distance between sampling location and the nearest OW/OA composter and whether this composter was in a rural or urban location. Increasing distance between sampling location and the nearest OW/OA composter was significantly associated with a decrease in the number of spores grown from the sample, which is consistent with the drop off in spore numbers previously observed with increasing distance from OW/OA composter^{61,187}. Samples that were closest to an urban OW/OA composter grew significantly fewer *A. fumigatus* colonies than samples closest to a rural OW/OA composter, which is likely an indication of land cover in the region of the sampling location. If the nearest composter is in an urban location, then it is likely that the sampling location is heavily urbanised and therefore further from woodlands with decomposing leaves or arable land with compost and decomposing crop residue. It is also possible that the likelihood of an airborne *A. fumigatus* spore settling, germinating and then releasing more spores is greater in a rural setting than in an urban setting, where concrete or tarmac surfaces inhibit spore germination.

None of the environmental variables included in the association analyses were found to have a significant effect on whether a sample grew ARAf or the number of ARAf colonies grown from a sample. While it is possible that none of these environmental variables affect development or distribution of ARAf, it is also possible that this analysis lacked sufficient power to detect associations due to the relatively small number of samples that grew ARAf (73 of 1,894 samples; 4%) or the limited number of colonies that were azole-resistant (111 of 2,366; 5%). This makes a case for citizen science air sampling for viable *A. fumigatus* spores to be repeated on a larger scale, involving more participants and/or more regular sampling

dates, to collect more ARAf from more locations in the UK; thereby increasing statistical power to detect associations.

The tebuconazole-resistant isolates collected in the first air sampling round (prefix SS18) were whole-genome sequenced as part of an ongoing genotyping project in Fisher lab headed by Dr Johanna Rhodes. The phylogenetic analysis grouped these isolates into three clusters: two large clusters corresponding to 'Clade A' and 'Clade B' previously described by Sewell *et al.* (2019)¹⁹⁷ and a smaller cluster corresponding to 'Clade Aa' previously described by Rhodes *et al.* (2021)²³¹. In all clusters, the UK and global SS18 isolates were mixed with the UK environmental and clinical isolates collected between 2005 and 2017, which demonstrates the panmictic nature of *A. fumigatus*. This clustering also demonstrates the clinical relevance of the airborne ARAf collected in this thesis as they are closely-related to isolates being recovered from patients with aspergillosis.

One limitation of this citizen science approach to sample collection is the assumption that participants left their air samplers exposed for 6-10 hours, on an outdoor windowsill, during daytime, as requested. Even this timeframe introduces variation between samples as a sampler left exposed for 10 hours has had nearly twice the amount of time to collect spores than a sampler exposed for six hours. If the citizen science project were to be repeated, this could be changed to approximately eight hours to ensure growth of *A. fumigatus* or number of *A. fumigatus* colonies grown is not a product of exposure time. Furthermore, it is likely that rain landing on the samplers would reduce their stickiness and could either wash off deposited spores or stop additional spores from sticking, therefore it is assumed that participants did not sample on rainy days, as requested. Finally, buildings are known to disrupt air flow²⁴⁰; therefore, attaching air samplers to windowsills may have resulted in fewer spores being collected than if the samplers had been exposed in open spaces or on rooftops.

The key findings of this chapter were that ~5% of *A. fumigatus* spores circulating in UK air were resistant to tebuconazole, ~4% were also resistant to at least one of the medical azoles ITZ, VCZ, PCZ and ISZ and ~1% were pan-azole resistant. The frightening reality of this is that an individual at risk of developing aspergillosis has a 1 in 25 chance of being exposed to a drug-resistant spore, which (if it establishes) may require treatment with more toxic drugs and reduce the individual's survival if the infection progresses to invasive aspergillosis (IA). Season, proximity to OW/OA composters and proximity to arable land were all identified in

this chapter as having an effect on the likelihood and intensity of exposure to *A. fumigatus* spores and further research is warranted to confirm these findings on a larger scale. The growing population of individuals at risk of aspergillosis in the UK, including those on immunosuppressants, receiving chemotherapy or who have suffered from severe influenza or COVID-19 infections, need to be identified and advised as to how to reduce their risk of exposure to *A. fumigatus* spores. This advice might include not living or working close to an OW/OA composter or arable fields, or not spending time outside when nearby farmers are spreading manure, compost or slurry on their fields. The finding that a number of airborne spores were resistant to all medical azoles currently used to treat aspergillosis highlights the need for development of new antifungals and their ringfencing for therapeutic use only. As the sampling approach used in this chapter is economical and straightforward to organise, it could be run on a long-term basis, regularly or every day, to further characterise fluctuations in baseline spore concentrations across space and time.

4.5. Acknowledgements

I would like to thank Kathleen Richards for her assistance with data entry and to Joshua Jackson and Christopher Edwards for their assistance with laboratory administration following the first air sampling round (SS18). Thank you to Professor Elizabeth Johnson and Professor Andy Borman of the PHE Mycology Reference Laboratory for letting me conduct CLSI susceptibility testing to medical azoles in their labs and to Dr Mark Fraser for conducting the MALDI-TOF spectrometry analysis on several isolates. Thank you to Asmaa Alghamdi of the University of Nottingham and Dr Jianhua Zhang of Wageningen University in the Netherlands for sharing primer details with me for the *cyp51A* promoter and coding regions, respectively. Thank you to Samuel Hemmings for extracting DNA from the tebuconazole-resistant SS18 isolates for WGS and to Dr Johanna Rhodes for processing the raw WGS reads, calculating the SNPs and running the phylogenetic analysis that I have included in this chapter. Thank you to Dr Pippa Douglas for providing the locations of composters in England with open windrow or outdoor activity. Thank you to Dr Christopher Uzzell for helping me to download environmental data and the UKCEH Land Cover maps, and for showing me how to use QGIS software to create maps and process these data.

Chapter 5: Azole-Resistant *Aspergillus fumigatus* Spores in UK Soil

The manuscript for this chapter has been uploaded to the preprint server bioRxiv with the intention of submitting to the American Society of Microbiology journal 'Applied and Environmental Microbiology': <https://www.biorxiv.org/content/10.1101/2021.07.08.451577v1>

Abstract

Compost has long been recognised as an ecological niche for *Aspergillus fumigatus* due to its role as a decomposer of organic matter and its ability to survive the thermophilic stages of the composting process, which kills off less thermotolerant microbes. Furthermore, composting facilities are associated with high levels of *A. fumigatus* spores, which are aerosolised from compost and cause respiratory illness in workers. As gardening is an occupation enjoyed by individuals of all ages in the UK, it is likely that they are being exposed to *A. fumigatus* spores from the composting of garden waste. Moreover, the risk of exposure to azole-resistant *A. fumigatus* spores might greatly increase when handling either commercially-produced compost or compost they have produced themselves.

In this chapter, 5,174 *A. fumigatus* were cultured from soil samples collected by citizen scientists from their gardens across the UK. Of these, 736 (14%) were resistant to tebuconazole and 553 (11%) were found to contain the known *cyp51A* resistance mechanisms TR₃₄/L98H or TR₄₆/Y121F/T289A associated with elevated minimum inhibitory concentrations to medical azoles. Additional *cyp51A* resistance mechanisms detected were TR₅₃, TR₄₆/Y121F/T289A/S363P/I364V/G448S and (TR₄₆)²/Y121F/M172I/T289A/G448S that are also associated with cross-resistance to medical azoles. Four isolates contained a novel TR₁₃₀/D430G resistance mechanism that is currently unpublished.

Association analyses found that soils sampled from garden locations containing compost – either commercial or homemade – were more likely to grow *A. fumigatus* and azole-resistant *A. fumigatus* (ARAF) than samples collected from borders, pots or planters without compost added. Furthermore, soil samples containing compost grew significantly greater numbers of *A. fumigatus* colonies than those without.

These findings highlight compost as a significant health hazard to individuals with pre-disposing factors to aspergillosis, and a potential health hazard to immunocompetent individuals who could be exposed to sufficiently high numbers of spores to develop aspergillosis infection. This raises the question of whether commercial producers should be responsible for sterilising compost of *A. fumigatus* spores before shipping, or whether compost bags should carry additional health warnings regarding inhalation of *A. fumigatus* spores. These results support increasing public awareness of the hazard posed by compost and measures that can be taken to reduce the exposure risk.

5.1. Introduction

5.1.1 Land use in the UK

The relationship between microbes in the soil and the surrounding environment is highly dependent on land use. In England, as of April 2018, 91.5% of land was classed as “non-developed”, which encompasses land used for agriculture, forest, open land, water, parks and gardens, and 8.3% was developed for industry, commerce, transport and residential use²⁴¹. The greatest use of land in England was for agriculture, which accounted for 62.8%²⁴¹. Agricultural land used for arable purposes is regularly tilled prior to sowing of seeds, causing disruption to fungal networks in the soil²⁴² and the aerosolisation of fungal spores²⁴³. Here, the spraying of crops to protect against fungal pathogens results in selection pressure on environmental fungi such as *Aspergillus fumigatus*²⁴⁴. Agricultural land grazed by farm animals is likely to be untouched by fungicides. However, the storage of manure²⁴⁵ and soiled bedding²⁴⁶ provide ideal growing conditions for *A. fumigatus* and might also present a selection pressure if the manure is from animals that have been treated for fungal disease or the bedding is residue from a crop sprayed with azoles.

There are currently 16 fungicides approved for use on turf grass in the UK including three azoles: propiconazole, epoxiconazole in combination with carbendazim, and tebuconazole in combination with prochloraz²⁴⁷. Turf is laid on golf courses, sports pitches, in parks, public gardens and private gardens²⁴⁸ that, combined, accounted for 7% of England’s 2018 land use²⁴¹. The UK is the second largest trade partner of the Netherlands for horticulture and imported £1.2 billion of live trees, plants and flowers in 2019²⁴⁹, which includes flower bulbs

that are dipped in azole fungicides¹²⁶ and then planted in public parks and gardens and residential gardens. As a saprotroph that is ubiquitous from the dispersal of its spores in the air, *A. fumigatus* is present on dead plant matter from all land uses when it is sent for composting – from fallen leaves to crop residues, manure, soiled animal bedding, discarded plants and bulbs and lawn cuttings – and it is exposed to the residues of every fungicide that has been applied to them.

5.1.1. Composting practices in the UK

As mentioned in Chapter 4, the UK government has committed to diverting waste from landfill and incineration to composting in order to reduce carbon dioxide (CO₂) emissions²¹⁴. The Environment Agency's protocol for producing quality compost from biodegradable waste allows input materials from agriculture, horticulture, aquaculture, forestry, hunting, fishing, brewing, baking, wood, pulp and paper processing, leather, fur and textiles industries, plastic and rubber manufacture, construction, households, gardens, parks and markets, as well as accepting soil and treated sewage sludge²⁵⁰. In 2018, there were 171 composting facilities in the UK certified by the Compost Certification Scheme for producing quality compost²⁵¹. These facilities processed 3.7 million tonnes of input materials per annum and produced 1.86 million tonnes of compost; of which 78% was sold for agriculture and horticulture, 9% for domestic or professional horticulture, 10% for land restoration and landscaping and 3% for forestry²⁵¹. Compost is recognised as an important source of organic matter and plant-available nutrients and features in the UK government's Food 2030 strategy for improving soil quality and maintaining the productive capacity of soil²⁵²; however, it is also acknowledged that the application of compost must not be harmful to the environment or human health²⁵³. Deportes *et al.* (1995) reviewed these potential harmful effects and highlighted the risks as: 1) accumulation of heavy metals and/or organic compounds in the food chain when compost is applied to agricultural land, 2) exposure to gastrointestinal pathogens found in sewage sludge through ingestion of compost, and 3) inhalation of endotoxins, bacteria and fungi, such as *A. fumigatus*, in dust produced when handling compost²⁵⁴.

5.1.2. Occupational risk of chronic aspergillosis

Occupations that have the greatest interaction with decomposing organic matter – waste collectors, workers at composting facilities and farmers – are recognised as those at the greatest risk of occupationally-acquired respiratory illness: in particular severe asthma with fungal sensitisation (SAFS), rhinitis, rhinosinusitis, hypersensitivity pneumonia and allergic bronchopulmonary aspergillosis (ABPA) from exposure to *Aspergillus*²⁵⁵. Other high-risk occupations include sawmill workers, mushroom processors and individuals handling grain, feed, hay or straw²⁵⁵ as these materials can emit fungal spore concentrations of 10^8 colony forming units per m^3 (CFU/ m^3) when disturbed²⁵⁶. Viegas *et al.* (2017) sampled for *Aspergillus* species in different occupational settings in Portugal and found the highest prevalence in wastewater treatment plants, followed by composting facilities, poultry feed stores, cork manufacturers, slaughterhouses and swineries²⁵⁷.

Studies conducted on domestic waste collectors and workers at composting facilities report increased eye and nose irritation, elevated IgE levels to allergens and moulds, reduced forced vital capacity (FVC) and increased prevalence of coughing, chronic bronchitis, rhinitis, asthma and chronic obstructive pulmonary disease (COPD)^{258–263}. Specific cases of chronic *Aspergillus*-related respiratory illness reported in waste handlers include a 29-year-old male in Germany²⁶⁴ and 35-year-old and 43-year-old males in the UK²⁶⁵ diagnosed with ABPA after collecting garden waste, and hypersensitivity pneumonitis in German²⁶⁶ and Belgian²⁶⁷ compost workers.

Farmers are at increased risk of respiratory illness due to their prolonged and repeated exposure to mouldy hay and straw, manure and decomposing vegetation and several studies have reported the same respiratory symptoms in farmers as those experienced by compost workers^{268–271}. A Danish study reported higher antibody levels to *A. fumigatus* antigens in farmers compare to city-dwelling controls²⁷²; a Finnish study found fungal spore counts, predominantly *Aspergillus* species, of between 10^4 - 10^7 CFU/ m^3 in dairy farm buildings²⁷³ and a Portuguese study reported asthma, wheezing and dyspnea in poultry and swine farm workers likely due to *Aspergillus* exposure²⁷⁴. Farmer's lung is a type of hypersensitivity pneumonitis caused by inhalation of allergens from mouldy hay. It is often attributed to

thermophilic bacteria but there have been cases attributed to *Aspergillus* species^{275,276} and several farmers have been diagnosed with ABPA in addition to farmer's lung^{277–279}.

5.1.3. Compost and gardening in the UK

In 2017, UK households spent approximately £450 million on compost²⁸⁰. Parks, public gardens and residential gardens are estimated to use 2-5% of the total compost produced in the UK and apply it more liberally at 300 tonnes per hectare (t/ha) than the 50 t/ha applied to agricultural land²⁵⁴. Furthermore, over one-third of households with access to a garden reported composting their garden and/or kitchen waste²⁸¹. The likelihood of these home composting setups containing azole residue is relatively high if the garden waste contains ornamental plants, bulbs or lawn clippings that have been treated with fungicides – as one-third of fungicides sold contain azoles – or if the household waste contains grains, fruits, vegetables, berries or vines protected by fungicides against mildews and rusts²⁸². This means that gardeners working with commercial or home composts are at risk of inhaling *A. fumigatus* spores that are aerosolised each time the compost is handled, and may be at further risk of inhaling *A. fumigatus* spores that are azole-resistant due to the co-occurrence of *A. fumigatus* and azole residues on organic waste. There has been a case described of a man developing hypersensitivity pneumonitis after working with home composted garden clippings, which was coined “residential composter's lung”²⁸³.

According to the Office of National Statistics, there were approximately 520,000 hectares of residential gardens in Great Britain (GB) in 2019, which constituted 29.5% of the total urban area²⁸⁴. In a survey of 7,715 residents in England, 14% of 16–24-year-olds, 43% of 25–44-year-olds, 64% of 45–64-year-olds, 72% of 65–74-year-olds and 58% of 75+-year-olds reported spending their free time gardening²⁸⁵. Assuming that most individuals with a garden or who report spending their free time gardening have worked with commercial and/or home compost, this puts a significant proportion of the population at-risk of inhaling large numbers of potentially azole-resistant *A. fumigatus* spores.

5.1.4. Aspergillosis from gardening

The main risk factors for invasive aspergillosis (IA) infections are having lung damage caused by infections or chronic lung disease or being immunocompromised, such that *A. fumigatus*

spores germinate in a lung cavity and cross into the bloodstream, resulting in sepsis or respiratory failure if not treated. There have, however, been several cases of individuals with no known underlying health conditions developing IA following exposures to huge numbers of *A. fumigatus* spores during gardening activities.

In Denmark, a 33-year-old male and 63-year-old gardener were admitted to hospital with *A. fumigatus* lung infections after spreading damp tree-bark chippings; the infection proved fatal for the 33-year-old but the 63-year-old recovered following itraconazole therapy²⁸⁶. The authors recommended that individuals with known immune deficiency avoid working with tree-bark products and that healthy individuals should not work with these products while they are wet and mouldy²⁸⁶. In France, a 28-year-old man reported inhaling dust while gathering laurel leaves in his garden and died ten days later from IA²⁸⁷. The authors suggest that aspergillosis should be suspected if an immunocompetent young person is hospitalised with community-acquired pneumonia of unknown origin, especially if they report inhaling vegetal dust²⁸⁷. In Germany, an immunocompetent 54-year-old female presented with respiratory failure after spreading tree-bark in her garden; *A. fumigatus* was cultured from her bronchoalveolar lavage fluid (BALF) and she recovered following treatment with voriconazole²⁸⁸. In the UK, an immunocompetent 47-year-old male died of IA after breathing in dust from the mulch he was spreading in his garden²⁸⁹, and a 34-year-old healthy male died of IA who had no predisposing factors apart from having worked as a gardener for 14 years²⁹⁰.

The National Aspergillosis Centre advises people to take care when opening bags of compost and handling rotting material, and recommends working outside on windy days when spores will be dispersed and wearing a facemask whilst doing so to avoid dust inhalation. Despite the recognised risks of handling compost, the only health warning printed on commercially-available compost bags is to not handle compost without gloves if you are pregnant to prevent toxoplasmosis infection²⁹¹.

5.1.5. *A. fumigatus* and azole-resistant *A. fumigatus* in UK soils

Bromley *et al.* (2014) collected soil samples from multiple sites in Manchester city centre and winter wheat, oilseed rape and winter barley fields outside of Manchester with a known history of azole treatment¹³². The urban soils grew 106 *A. fumigatus* CFU per gram (CFU/g) on average compared to 43.5 CFU/g in agricultural soils. All isolates were tested for susceptibility

to itraconazole (ITZ), voriconazole (VCZ) and posaconazole (PCZ) and four isolates from agricultural soils were azole-resistant: two had no *cyp51A* polymorphisms and two contained TR₃₄/L98H. Interestingly, the modal minimum inhibitory concentrations (MICs) for VCZ and PCZ were lower in isolates from agricultural soils (0.5 mg/L and 0.06 mg/L, respectively) than in isolates from urban soils (1 mg/L and 0.125 mg/L, respectively). These differences were statistically significant for PCZ but not for VCZ, and no differences were observed for ITZ.

Dunne *et al.* (2017) collected soil samples from five sites around a hospital building in Dublin, Ireland and commercial compost, as well as swabbing the surface of flower bulbs purchased from a garden centre in Dublin that were imported from the Netherlands¹³³. They do not state how many *A. fumigatus* isolates grew from the hospital and compost soils but all were tested to ITZ, VCZ and PCZ: one isolate from commercial compost was resistant to VCZ and contained TR₄₆/Y121F/T289A and one isolate from the hospital soils was pan-azole resistant with TR₃₄/L98H. Of the 19 isolates grown from flower bulbs, seven (37%) were resistant to at least one azole: four isolates contained TR₄₆/Y121F/T289A and three contained TR₃₄/L98H.

Tsitsopoulou *et al.* (2018) collected 671 soil samples from urban and rural locations in South Wales, which grew a total of 496 *A. fumigatus* isolates, and 30 (6%) isolates were azole-resistant when tested for susceptibility to ITZ, VCZ and PCZ¹³⁴. The 419 samples collected from arable fields grew 288 isolates of which 15 (5.2%) were azole-resistant, the 35 samples collected from non-public areas (gardens and allotments) grew 28 isolates of which none were resistant, and the 216 samples collected from public areas (public gardens, parks, hospital grounds and botanical gardens) grew 179 isolates of which 15 (8.4%) were resistant. The 12 compost samples grew 10 isolates, none of which were resistant.

Sewell *et al.* (2019) collected 178 soil and compost samples from several sites across South West England, including public gardens, parks, cemeteries, flower beds outside hospitals, a forest, a lavender farm and farmland¹³¹. 131 (78%) samples grew *A. fumigatus* and 11 (6%) samples grew 83 isolates that were resistant to at least one azole when tested for susceptibility to ITZ, VCZ and PCZ. Of the 19 azole-resistant isolates sequenced for *cyp51A*, six isolates contained TR₄₆/Y121F/T289A and 13 isolates contained TR₃₄/L98H.

Fraaije *et al.* (2020) collected soil samples from experimental cropland with no or known azole treatment in the UK, as well as from commercial wheat fields in the UK, Germany and the

Netherlands and arable crops, woodlands and grass verges in Hungary, Austria, Germany, Belgium and the Netherlands⁸⁹. The soils grew 0-10 *A. fumigatus* CFU/g, and all isolates were tested for susceptibility to azole fungicides ITZ, VCZ, imazalil and tebuconazole. A total of 302 isolates were grown from soils collected from experimental crop fields in the UK of which 27 (8.9%) were resistant to at least one azole. A further 140 isolates were grown from commercial wheat fields in the UK, of which 17 (12.1%) were resistant to at least one azole. The polymorphisms found in *cyp51A* for the resistant isolates were: none, D262Y, F46Y/M172V/E427K, TR₃₄/L98H and TR₄₆/Y121F/T289A.

Van der Torre *et al.* (2020) collected 74 soil samples adhered to root vegetables from farms and private gardens across the UK, which grew 146 *A. fumigatus* isolates¹⁹². These isolates were tested for susceptibility to ITZ, VCZ and PCZ and none were found to be azole-resistant, although six isolates were pan-azole resistant that were identified as members of *Aspergillus* Section *Fumigati*.

These studies, combined, have collected large numbers of soil samples from urban and rural locations in England, Wales, and Ireland to culture *A. fumigatus* isolates and test them for susceptibility to the medical azoles ITZ, VCZ and PCZ. The prevalence of azole-resistant *A. fumigatus* has ranged from 0% in soil samples from root vegetables¹⁹² and compost¹³⁴, 5.2-12.1% in arable soils^{89,134}, 8.4% in soils from public areas¹³⁴ and 37.0% from the outside of bulbs imported from the Netherlands¹³³.

Given that composting facilities are known sources of *A. fumigatus* spores and that *Aspergillus*-related illnesses are reported in compost workers and farmers, as well as in immunocompetent individuals following gardening activities, it is likely that commercial and homemade composts contain high numbers of *A. fumigatus* spores. Furthermore, given the likelihood of azole residues occurring on organic matter that is composted, both commercially and at-home, there is also the opportunity for azole-resistant *A. fumigatus* (ARAF) to develop. Existing studies that have collected *A. fumigatus* from private gardens have not detected resistance but the numbers of isolates cultured were relatively small: Tsitsopoulou *et al.* (2018) cultured 26 isolates from 33 soil samples collected from private gardens¹³⁴ and Van der Torre *et al.* (2020) cultured 146 isolates from 74 soil samples collected from farms and private gardens¹⁹².

The aims of this chapter were fourfold:

1. to ascertain numbers of *A. fumigatus* and ARAf spores in soil collected from residential gardens in the UK and compare these to numbers in soil samples collected elsewhere in the UK by existing studies,
2. to establish whether compost (commercial or homemade) contain more *A. fumigatus* and/or ARAf than non-enriched garden soils,
3. to determine whether other environmental variables influence spore numbers or azole-resistance in soil, and
4. to characterise *cyp51A* polymorphisms in soilborne ARAf spores.

These aims were achieved by culturing *A. fumigatus* from the soil samples collected by citizen scientists from borders, plant pots, compost heaps and bags of compost/manure in their garden (Chapter 3).

5.2. Methods

5.2.1. Recruitment for citizen science sampling project

As detailed in 3.2.4, citizen scientists were recruited to take part in the project *via* posts on social media platforms Twitter and Facebook, posters displayed at Imperial College London and UK Centre for Ecology & Hydrology (UKCEH) and a blog on the UKCEH website (Appendix 7). In total, 334 individuals registered their interest in taking part and were sent sampling packs containing two sample bags, simple instructions (Appendix 8), a questionnaire (Appendix 9) and a Freepost return envelope, and 246 individuals participated. This 74% return rate, much higher than the 10-15% return rate generally expected for citizen scientist projects (personal comms, CEE Symposium Citizen Science, 14th June 2018) suggests that participants were motivated to take part and that the sampling strategy was straightforward and/or well-explained.

5.2.2. Processing soil samples, culturing *A. fumigatus* and testing for ARAf

When envelopes were received back from citizen scientists, the soil samples and paperwork were processed as detailed in section 2.5. Briefly, 2 g of each soil sample was suspended in 8

ml of buffer (0.85% NaCl and 0.01% Tween 20 in diH₂O) and the surface 200 µl was pipetted onto two Sabouroud dextrose agar (SDA) plates: one containing penicillin (200 mg/L) and streptomycin (400 mg/L) and the other containing penicillin (200 mg/L), streptomycin (400 mg/L) and tebuconazole (6 mg/L). After incubation for 48 hours, the number of *A. fumigatus* isolates grown on each plate were recorded and isolates on the tebuconazole plates were picked into 1.5 ml tubes containing 200 µl of mould preservation solution (0.2% agar and 0.05% Tween 20 in diH₂O) and stored in the fridge.

5.2.3. Cryopreservation, DNA extraction and gene sequencing of *ARAf*

Isolates that grew on the SDA plates containing tebuconazole were cryopreserved at -80°C (section 2.6) and had their DNA extracted and stored at -20°C (section 2.7). The DNA from these isolates was then amplified and sequenced for the *cyp51A* gene promoter and coding regions to determine the polymorphisms causing resistance (section 2.9).

5.2.4. Confirming identity as *A. fumigatus*

If an isolate failed to amplify with primers for the *cyp51A* gene promoter and coding regions it had its beta-tubulin gene sequenced to confirm or disprove its identity as *A. fumigatus*.

5.2.5. Environmental factors relating to UK sampling dates and locations

Variables that were provided by the citizen science participant were the geographical location of the sample, where in the garden it was collected from (border, plant pot, compost heap or bag of compost/manure) and the date on which it was collected. Additional data were obtained for all samples, including maximum daily temperature on sampling date at sampling location (section 2.12), land cover classification of sampling location (section 2.13) and percentage of arable land in 2 km surrounding sampling location (section 2.14). Maximum daily temperatures at sampling locations were included as a variable to determine whether ambient temperature affected the viability of *A. fumigatus* or *ARAf* spores. The distance from all UK sampling locations to the nearest composting facility with open windrow or outdoor activity (OW/OA) was calculated (section 2.15). The land cover classification for each OW/OA

composting facility was also obtained to ensure any association between a sample growing *A. fumigatus* or ARAf and distance to its nearest OW/OA composter was not confounded by the composter's land cover.

5.2.6. Generalised linear models

	Name of variable	Type of data	Number of samples included
Dependent variables	Growth of <i>A. fumigatus</i>	Binary	501 ^a
	Number of <i>A. fumigatus</i> grown	Count	327 ^b
	Growth of ARAf	Binary	509
	Number of ARAf grown	Count	101 ^b
Independent variables	Garden location sample collected from	Categorical	509
	Date sample collected	Categorical	509
	Maximum daily temperature at sampling location	Continuous	509
	Land cover classification of sampling location	Categorical	509
	Urban or rural sampling location	Categorical	509
	Percentage arable land in 2km buffer surrounding sampling location	Continuous	509
	Distance to nearest OW/OA composter	Continuous	509
	Land cover classification of nearest OW/OA composter	Categorical	509
	Urban or rural location of nearest OW/OA composter	Categorical	509

Table 5.1: Dependent and independent variables used in generalised linear model fitting to determine the influence of climate and location on growth of *A. fumigatus* and azole-resistant *A. fumigatus*. ^a It was unknown for eight samples whether *A. fumigatus* grew or not due to contamination. ^b Counts of 0 were excluded to avoid replicating the logistic regressions. OW/OA composter = composter with open windrow or outdoor activity.

The dependent and independent variables used in fitting the generalised linear models (GLM) are shown in Table 5.1. Logistic regressions were used to look for associations between these independent variables and the likelihood of a sample growing *A. fumigatus* or ARAf. Poisson regressions were deemed unsuitable for analysis due to overdispersion of the count data, shown in Figure 5.1, which resulted in the residual deviance in the model being far greater than degrees of freedom when these should be in approximately 1:1 ratio. Negative binomial regressions were used instead to determine whether any of the independent variables significantly affected the number of *A. fumigatus* or ARAf colonies grown from a sample. The peaks at 30 and 50 colonies in the histogram in Figure 5.1 are due to the difficulties in counting exact numbers of *A. fumigatus* or ARAf colonies on plates that were overgrown with colonies, such that plates were recorded as having >30 or >50 colonies.

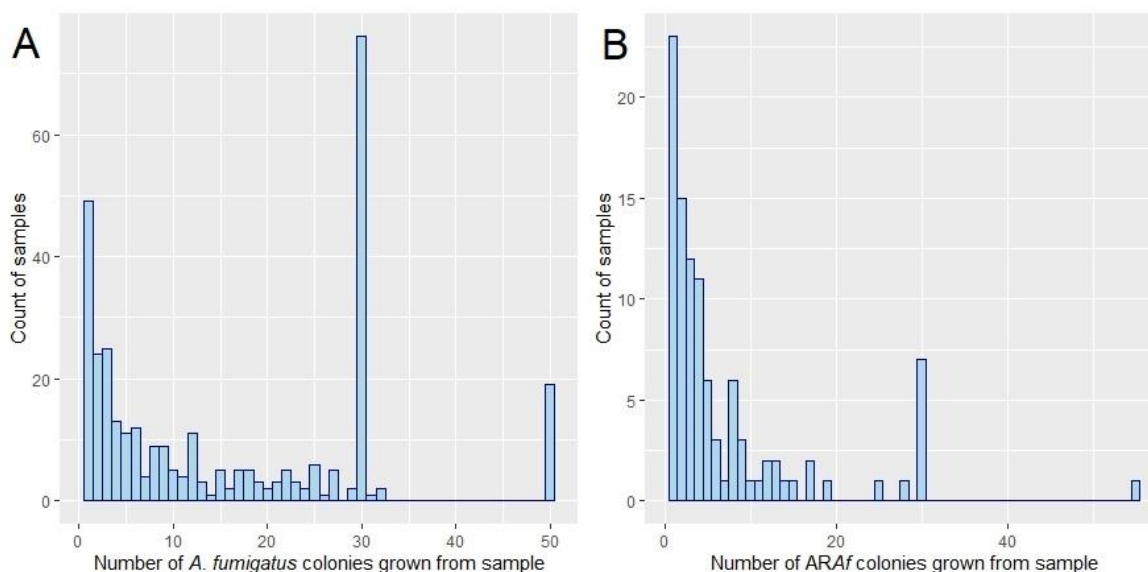


Figure 5.1: Histograms showing the distribution of numbers of A) *A. fumigatus* colonies and B) azole-resistant *A. fumigatus* colonies grown from positive samples.

5.3. Results

5.3.1. *A. fumigatus* and ARAF in UK soil samples

In total, 509 soil samples were collected and sent in by citizen scientists in the UK of which 327 (64%) grew 5,174 *A. fumigatus* isolates and 101 (20%) grew 736 ARAF isolates (Table 5.2 and 5.3). The majority of samples, 451 of 509 (89%), were assigned a single location in the garden that they had been collected from: either a border, bag of compost, bag of manure, compost heap or pot/planter. For the remaining samples, multiple locations had been assigned by the participant or it was apparent from the sample's description that a single location was not appropriate. These multiple locations occurred for soils collected from borders or pots/planters recently covered or topped up with compost or manure. Across all samples that grew *A. fumigatus* the average CFU/g was 316, which ranged from 0 CFU/g in the sample collected from a border plus manure bag to 600 CFU/g in the sample collected from a manure bag (Table 5.3). Across all samples that grew ARAF the average CFU/g was 145, which ranged from 0 CFU/g in samples collected from several garden locations to 214 CFU/g in samples collected from compost heaps (Table 5.3). Figure 5.2 shows the geographical locations that samples were collected from, with samples that did not grow *A. fumigatus* shown in blue, samples that grew *A. fumigatus* shown in green and samples that grew ARAF shown in red.

Location in garden soil sample was collected from	Number of samples	Samples that grew <i>A. fumigatus</i> (% of samples)	Samples that grew ARAf (% of samples)
Border (B)	206	99 (48)	19 (9)
B + CB	7	4 (57)	1 (14)
B + CH	5	3 (60)	0 (0)
B + MB	1	0 (0)	0 (0)
B + MB + CH	1	1 (100)	0 (0)
Compost bag (CB)	49	44 (90)	20 (41)
Compost heap (CH)	80	58 (73)	27 (34)
Manure bag (MB)	1	1 (100)	0 (0)
Pot/planter (PP)	115	79 (69)	26 (23)
PP + CB	38	33 (87)	8 (21)
PP + CB + CH	3	2 (67)	0 (0)
PP + CB + MB	2	2 (100)	0 (0)
PP + CH	1	1 (100)	0 (0)
Total	509	327 (64)	101 (20)

Table 5.2: A breakdown of the number of soil samples collected, the number and percentage of soil samples that grew *A. fumigatus* and the number and percentage of samples that grew azole-resistant *A. fumigatus* (ARAf) by the location(s) in the garden that the soil sample was collected from.

Location in garden soil sample was collected from	Number of <i>A. fumigatus</i> isolates grown	Average <i>A. fumigatus</i> CFU/g ^a	Number of ARAf isolates grown (% of <i>A. fumigatus</i> isolates)	Average ARAf CFU/g
Border (B)	1,009	204	121 (12)	127
B + CB	56	280	8 (14)	160
B + CH	44	293	0 (0)	0
B + MB	0	0	0 (0)	0
B + MB + CH	1	20	0 (0)	0
Compost bag (CB)	993	451	137 (14)	137
Compost heap (CH)	1,464	505	289 (20)	214
Manure bag (MB)	30	600	0 (0)	0
Pot/planter (PP)	1,005	254	130 (13)	98
PP + CB	529	321	51 (15)	128
PP + CB + CH	21	210	0 (0)	0
PP + CB + MB	4	40	0 (0)	0
PP + CH	18	360	0 (0)	0
Total	5,174	316	736 (14)	145

Table 5.3: Numbers of *A. fumigatus* and azole-resistant *A. fumigatus* (ARAf) isolates grown from each garden location that soil samples were collected from, and the average colony-forming units per gram (CFU/g) for each location. ^a Calculated as the number of *A. fumigatus* isolates grown divided by the number of samples that grew *A. fumigatus*, multiplied by 20 to account for dilution factor of soil in buffer.



Figure 5.2: Maps showing locations of soil samples collected by citizen scientists. Blue dots indicate samples that did not grow *A. fumigatus* colonies, green dots indicate samples that did and red dots indicate samples that grew azole-resistant *A. fumigatus*.

5.3.2. *Cyp51A* polymorphisms in UK soilborne ARA^f

Of the 736 isolates that grew in the presence of tebuconazole at 6 mg/L, 93 (13%) failed to re-grow after short-term storage in the fridge. The remaining isolates were sequenced for *cyp51A* to determine the polymorphisms responsible for tebuconazole-resistance. The polymorphisms for each isolate, along with the soil sample's geographical and garden location, are shown in Appendix 14, and these results are summarised by garden location in Table 5.4.

<i>cyp51A</i> polymorphisms	Location in garden that soil sample was collected from						Total (%):
	B	B + CB	CB	CH	PP	PP + CB	
C270R					1		1 (0)
I242V					5		5 (1)
TR ₃₄				1	1	1	3 (0)
TR ₃₄ /L98H	82	7	117	237	65	34	542 (74)
TR ₃₄ /L98H/Q191E			1				1 (0)
TR ₃₄ /L98H/R196L		1					1 (0)
TR ₃₄ /L98H/K240R	1				1		2 (0)
TR ₃₄ /L98H/T289A/I364V/G448S				6			6 (1)
TR ₃₄ /L98H/K372R				1			1 (0)
TR ₃₄ /L98H/P394R			1				1 (0)
TR ₃₄ /L98H/F404C/F459S/A460S			1				1 (0)
TR ₃₄ /L98H/F404V			1				1 (0)
TR ₃₄ /L98H/N406D			1				1 (0)
TR ₃₄ /L98H/N406M				1			1 (0)
TR ₃₄ /L98H/K421R			1				1 (0)
TR ₃₄ /L98H/P443L					2		2 (0)
TR ₃₄ /L98H/A460S					1		1 (0)
TR ₃₄ /L98H/D481N			1			1	2 (0)
(TR ₃₄) ² /L98H	1						1 (0)
TR ₄₆ /Y121F/M178W/T289A/S363P/I364V/G448S				1			1 (0)
TR ₄₆ /Y121F/T289A			1	14		1	16 (2)
TR ₄₆ /Y121F/T289A/S363P/I364V/G448S				4			4 (1)
(TR ₄₆) ² /Y121F/M172I/T289A/G448S				1			1 (0)
TR ₅₃	1			1			2 (0)
TR ₁₃₀ /D430G				4			4 (1)
Wild-type	3			1	23		27 (4)
Failed to sequence	3			3	8		14 (2)
Isolate failed to re-grow	30		12	14	23	14	93 (13)
Total:	121	8	137	289	130	51	736

Table 5.4: The number of *A. fumigatus* and non-*A. fumigatus* isolates (failed to sequence) grown from soils collected from each garden location that were azole-resistant, and a summary of their *cyp51A* polymorphisms. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.

5.3.3. Maximum air temperatures on soil sampling dates

The 509 soil samples were collected from every country in the UK: 433 from England, 43 from Scotland, 27 from Wales and 6 from Northern Ireland. The soil sampling citizen project was advertised to take place on 21st June 2019, which was the summer solstice date for this year, but sample collection on any nearby date was encouraged for greater participation. As a result, 263 (52%) samples were collected on 21st June 2019 and the remaining 48% of samples were collected on 20 dates throughout June and July 2019. The maximum daily temperatures for sampling locations that were sampled from at the given date ranged from 12.5-30.5°C are shown in Figure 5.3.

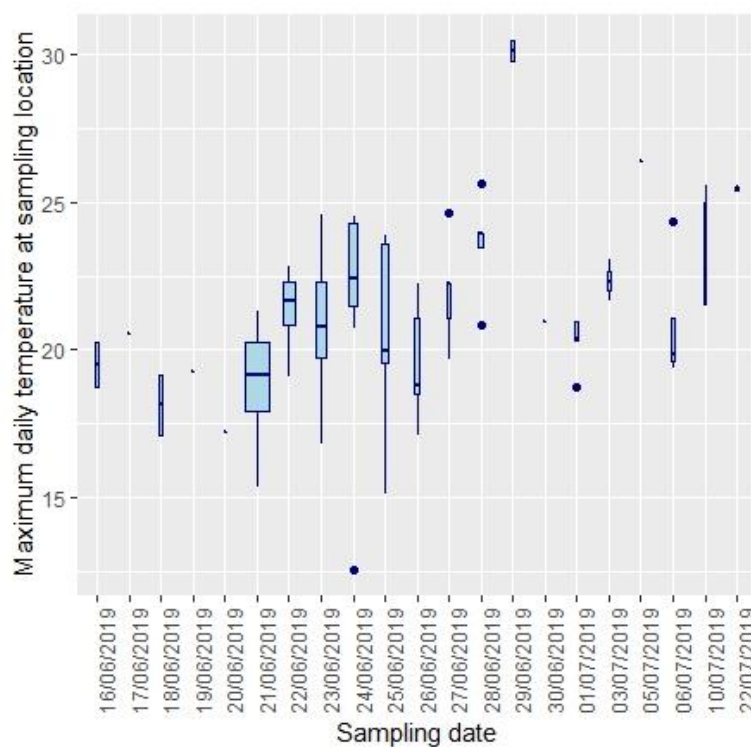


Figure 5.3: Boxplot of maximum daily temperature at sampling locations on the date that soil sample was collected.

5.3.4. Land cover classifications of sampling locations

The 509 soil samples were collected from 249 unique locations across the UK: 210 locations in England, 22 in Scotland, 14 in Wales and 3 in Northern Ireland (Figure 5.4). These locations were predominantly urban ($n = 230$; 92%), with the 19 rural locations classified as 1 (woodland), 3 (arable), 4 or 5 (grassland). The breakdown of land cover classes for sampling locations by country is shown in Table 5.5.

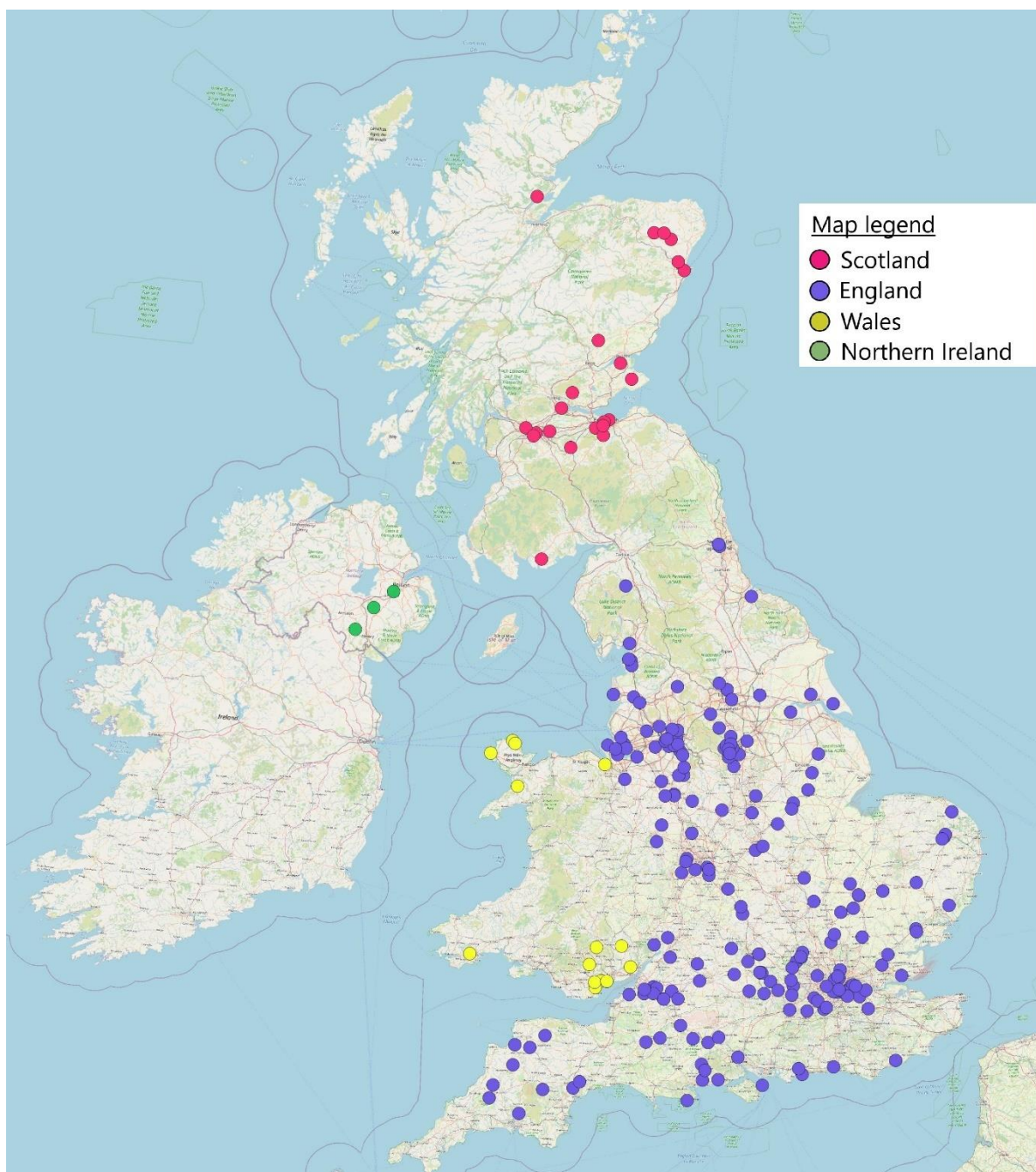


Figure 5.4: The 249 unique locations in the UK that soil samples were collected from, coloured by country.

Land cover classification	Countries				Totals
	England	Scotland	Wales	Northern Ireland	
1	2	0	0	0	2
3	2	4	0	0	6
4	7	1	1	1	10
5	0	0	0	1	1
Rural total (% of locations):	11 (5)	5 (23)	1 (7)	2 (67)	19 (8)
20	32	2	3	0	37
21	167	15	10	1	193
Urban total (% of locations):	199 (95)	17 (77)	13 (93)	1 (33)	230 (92)
Total:	210	22	14	3	249

Table 5.5: Breakdown of land cover classifications for soil sampling locations by country, obtained from UK Centre for Ecology & Hydrology's 2019 land cover maps for Great Britain and Northern Ireland.

5.3.5. Land cover classifications of composting facilities with open windrow or outdoor activity in the UK

The land cover classifications of the 257 OW/OA composting facilities in England, Scotland, Wales and NI are detailed in section 4.3.7 as the same sites were used in both analyses.

5.3.6. Distance from UK sampling locations to nearest composting facility

The 249 unique sampling locations across England, Scotland, Wales and NI were used to calculate distance to the nearest OW/OA composting facility. The closest distance between a sampling location and composting facility was 292 m and the furthest distance was 88 km. In total, nine sampling locations were within 2 km of an OW/OA composting facility. Figure 5.5A shows the distances between sampling locations and the closest OW/OA composting facility.

To determine the distance for any postcode in the UK to the nearest OW/OA composting facility, all current UK postcodes ($n = 1,773,498$) were downloaded and the distance between each postcode and the nearest OW/OA composter was calculated. The mean distance between all UK postcodes and the nearest OW/OA composter was 13 km and the median distance was 10 km. There were 11 postcodes that were 0 km from an OW/OA composting facility and 47,315 postcodes within 2 km. The furthest distance between a postcode and

nearest composter was 367 km. Figure 5.5B shows the distances between UK postcodes and the nearest OW/OA composting facility for the 1,768,554 postcodes that were within 90 km of the nearest composter, to use the same distance scale as Figure 5.5A.

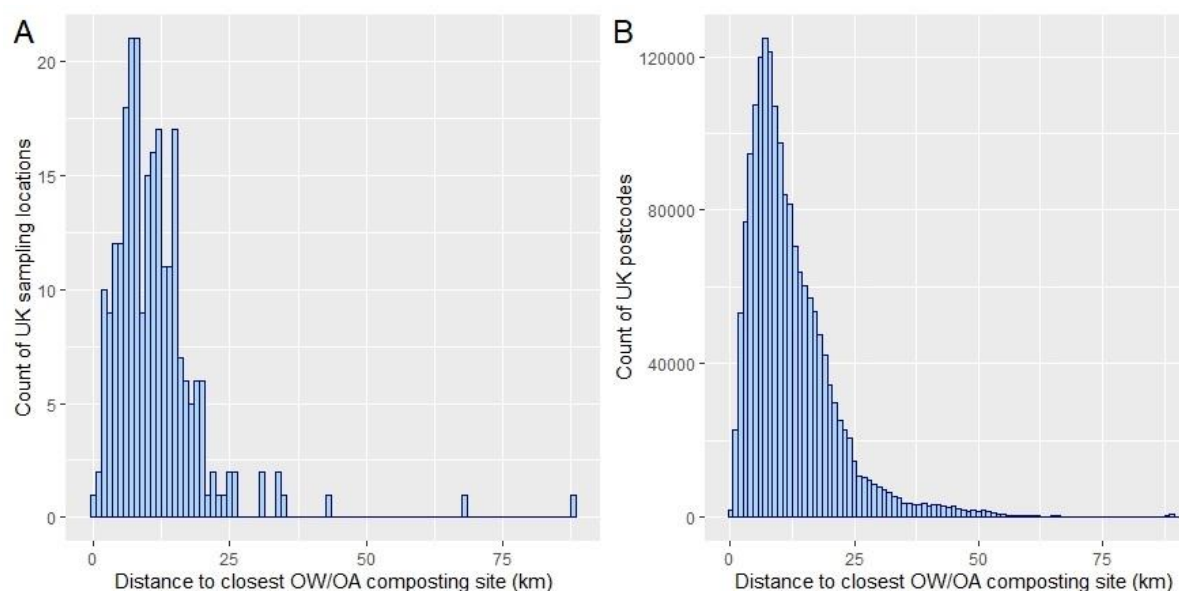


Figure 5.5: Histogram showing distances between A) UK soil sampling locations ($n = 249$) and B) all UK postcodes ($n = 1,768,554$) and the closest composting facility with open windrow or outdoor activity within 90 km.

5.3.7. Percentage of arable land in 2km buffer surrounding sampling locations

The percentage of arable land (land cover class 3) in a 2 km buffer around sampling location was calculated for 249 unique locations in England, Wales, Scotland and NI. Of these locations, 35 (14%) did not have any arable land within 2 km and the remainder had between 0.005% and 90%. As shown in Table 5.6, Wales had the highest proportion (29%) of sampling locations without arable land in the surrounding 2 km buffer and NI had no sampling locations without arable land in their surroundings.

Country	Number of unique sampling locations	Samples without arable land in 2 km buffer (% of samples)	Percentage of arable land in 2 km buffer of remaining sampling locations (%)
England	210	28 (13)	0.005-90
Scotland	22	24 (14)	0.4-70
Wales	14	23 (29)	0.4-28
Northern Ireland	3	0 (0)	1-10

Table 5.6: Breakdown by country of the percentage of arable land within surrounding 2 km of unique sampling locations.

5.3.8. Factors influencing whether samples grew *A. fumigatus*

5.3.8.1. Logistic regression model for UK samples

The logistic regression on UK samples ($n = 501$) to determine which factors influenced whether samples grew *A. fumigatus* excluded eight samples because the SDA plates without tebuconazole were too contaminated to count the numbers of *A. fumigatus* colonies. The only independent variable that significantly affected whether a sample grew *A. fumigatus* was the location in the garden from which the sample was collected ($\chi^2 = 67.3$, $df = 12$, $p < 0.01$). The relationship between garden locations from which samples were collected and the growth of *A. fumigatus* from samples is shown in Figure 5.6.

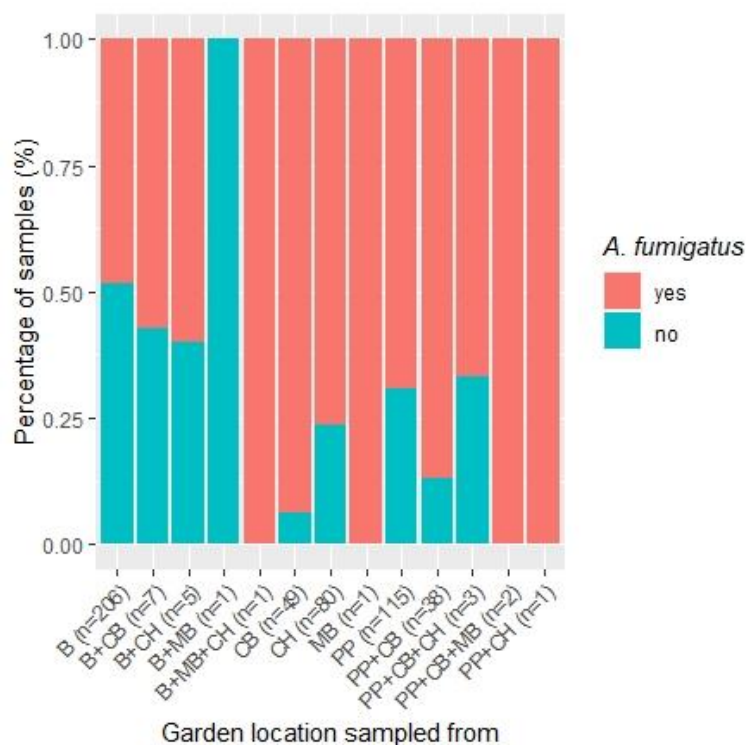


Figure 5.6: Plot showing the relationship between whether a sample grew *A. fumigatus* and the garden location from which the sample was collected. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.

The odds ratios and p-values for the locations in the garden from which the samples were collected are shown in Table 5.7. Samples collected from a compost bag, compost heap, pot/planter and pot/planter plus compost bag had significantly increased odds of growing *A. fumigatus* ($p < 0.01$) compared to samples collected from a border. There were no significant changes in odds of growing *A. fumigatus* from other sampling locations.

Independent variable	Odds ratio (95% CI)	Pr(> z)
Location in garden sampled from		
Border (baseline)		
B + CB	1.43 (0.31-7.40)	0.64
B + CH	1.61 (0.26-10.24)	0.61
B + MB	- ^a	0.99
B + MB + CH	- ^a	0.99
Compost bag	15.70 (5.50-66.19)	<0.01
Compost heap	3.45 (1.93-6.40)	<0.01
Manure bag	- ^a	0.99
Pot/planter	2.42 (1.50-3.95)	<0.01
PP + CB	7.07 (2.88-21.28)	<0.01
PP + CB + CH	2.14 (0.20-46.50)	0.53
PP + CB + MB	- ^a	0.98
PP + CH	- ^a	0.99

Table 5.7: Odds ratios, confidence intervals and p-values calculated for independent variables included in a logistic regression model using samples collected in the UK ($n = 501$) to explain whether a sample grew *A. fumigatus*. Significant results ($p \leq 0.05$) are highlighted in bold.
^aInsufficient data to calculate odds ratio and confidence intervals. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.

5.3.9. Factors influencing the number of *A. fumigatus* colonies grown from a sample

5.3.9.1. Negative binomial model for UK samples

In the negative binomial regression model run on UK samples that grew *A. fumigatus* ($n = 335$), the only independent variable that was found to have a significant effect on the number of *A. fumigatus* colonies grown was the location in the garden from which the sample was collected ($\chi^2 = 50.8$, $df = 11$, $p < 0.01$). The relationship between garden locations from which samples were collected and the number of *A. fumigatus* colonies grown from samples is shown in Figure 5.7.

Samples collected from several garden locations grew significantly more *A. fumigatus* colonies when compared to samples collected from borders: compost bag ($p < 0.01$), compost heap ($p < 0.01$) and pot/planter plus compost bag ($p = 0.02$). Samples collected from a pot/planter plus compost bag plus manure bag grew fewer *A. fumigatus* colonies than samples collected from borders, although this reduction was marginally significant ($p = 0.05$).

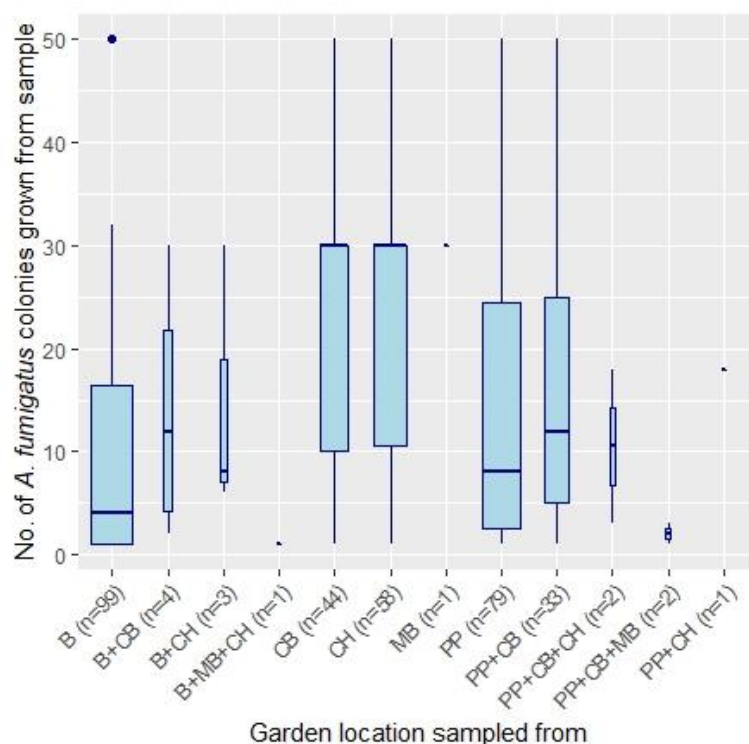


Figure 5.7: Plot showing the relationship between the number of *A. fumigatus* colonies grown from a sample and the garden location from which the sample was collected. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.

5.3.10. Factors influencing whether samples grew *ARAF*

5.3.10.1. Logistic regression model for UK samples

In the logistic regression model that included UK samples ($n = 509$) to determine which factors influenced whether a sample grew *ARAF*, location in garden that sample was collected from was the only independent variables tested that significantly improved upon the null model in explaining the outcome ($\chi^2 = 43.0$, $df = 12$, $p < 0.01$). The relationship between garden locations from which samples were collected and the growth of *ARAF* from samples is shown in Figure 5.8.

The odds ratios and p-values for the model, including the location in the garden from which the sample was collected, are shown in Table 5.8. Samples collected from a compost bag, compost heap, pot/planter and pot/planter plus compost bag had significantly increased odds of growing *ARAF* ($p < 0.01$) compared to samples collected from a border. There were no significant changes in odds of growing *ARAF* from other sampling locations.

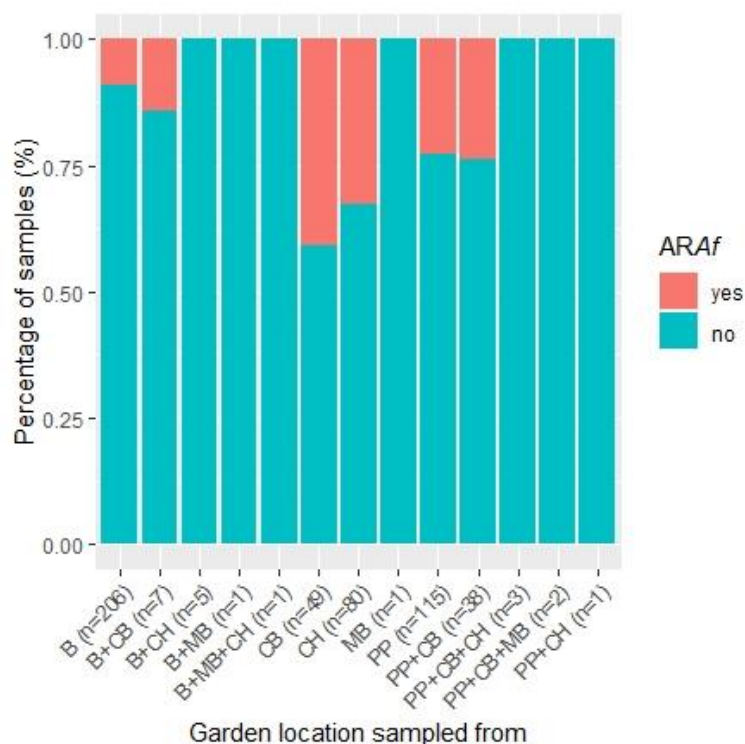


Figure 5.8: Plot showing the relationship between whether a sample grew azole-resistant *A. fumigatus* and the garden location from which the sample was collected. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.

Independent variable	Odds ratio (95% CI)	Pr(> z)
Location in garden sampled from		
Border (baseline)		
B + CB	1.64 (0.08-10.32)	0.65
B + CH	^a	0.99
B + MB	^a	0.99
B + MB + CH	^a	0.99
Compost bag	6.79 (3.25-14.37)	<0.01
Compost heap	4.74 (2.45-9.32)	<0.01
Manure bag	^a	0.99
Pot/planter	2.88 (1.52-5.53)	<0.01
PP + CB	3.05 (1.22-7.26)	<0.01
PP + CB + CH	^a	0.99
PP + CB + MB	^a	0.99
PP + CH	^a	0.99

Table 5.8: Odds ratios, confidence intervals and p-values calculated for independent variables included in a logistic regression model using samples collected in the UK ($n = 509$) to explain whether a sample grew azole-resistant *A. fumigatus*. Significant results ($p \leq 0.05$) are highlighted in bold. ^aInsufficient data to calculate odds ratio and confidence intervals. B=border, CB=compost bag, CH=compost heap, MB=manure bag, PP=pot/planter.

5.3.11. Factors influencing the number of ARAf colonies grown from a sample

5.3.11.1. Negative binomial model for UK samples

In the logistic regression model that included UK samples that grew ARAf ($n = 101$) to determine which factors influenced the number of ARAf colonies grown from samples, the independent variables that were found to have a significant effect were: land cover classification of nearest OW/OA composting facility to sampling location ($\chi^2 = 16.9$, $df = 7$, $p = 0.02$) and whether the nearest OW/OA composting facility was in an urban or rural location ($\chi^2 = 5.2$, $df = 1$, $p = 0.02$). The relationships between both significant independent variables and the number of ARAf colonies grown from samples are shown in Figure 5.9.

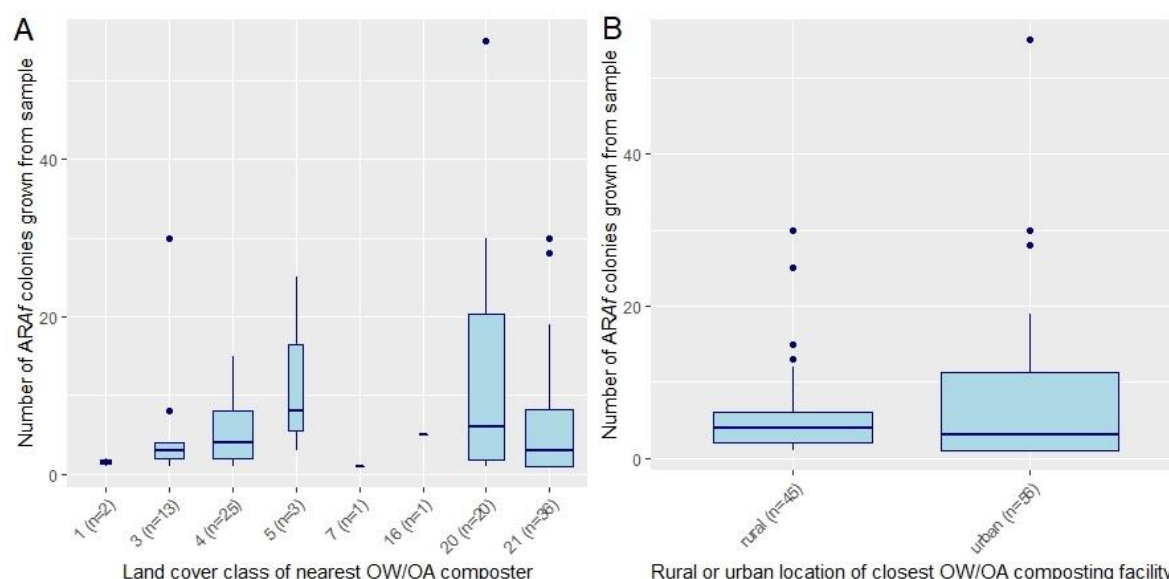


Figure 5.9: Plots showing the relationship between the number of azole-resistant *A. fumigatus* colonies grown from samples and A) land cover class of nearest composting facility with open windrow or outdoor activity (OW/OA) to sampling location, and B) rural or urban location of nearest OW/OA composting facility to sampling location.

The model with the lowest AIC included whether the nearest OW/OA composting facility was in an urban or rural location. In this model, samples that were closest to urban OW/OA composting facilities grew significantly higher numbers of ARAf colonies than samples closest to rural OW/OA composting facilities ($p = 0.02$).

County, north to south	Air sampling																Soil sampling			
	Summer 2018				Autumn 2018				Winter 2018				Spring 2019				Summer 2019			
	1		2		1		2		1		2		1		2		1		2	
	A	R	A	R	A	R	A	R	A	R	A	R	A	R	A	R	A	R	A	R
Inverness-shire					0	0			2	0			1	0			30	0	5	0
Fife	1	0			3	0			0	0			1	0			0	0	0	0
Midlothian	2	0	0	0													0	0	30	3
Dumfriesshire	0	0			0	0			7	1			0	0			1	1		
Lancashire	3	0	5	0									0	0			5	0	3	3
Lancashire													0	0			50	0	0	0
West Yorkshire					1	0	0	0	34	1	35	0	0	0	0	0	2	0	0	0
Lancashire									8	0	7	0	0	0	0	0	9	0	12	0
Greater Manchester	1	0			0	0			0	0			0	0			4	0	2	0
Greater Manchester													0	0	0	0	11	6		
Cheshire													0	0			0	0	0	0
Cheshire													1	0			0	0	0	0
Cheshire	2	0	0	0	2	0	0	0	2	0	1	0	0	0	0	0	22	0	50	17
Staffordshire					0	0			1	0			1	1			0	0	0	0
Norfolk													1	0			3	0	0	0
West Midlands					2	0			0	0			0	0	0	0	0	0	0	0
West Midlands	7	0	8	2	3	0	2	0	0	0	0	0	0	0	0	0	30	0	0	0
West Midlands	0	0	1	0													3	0	22	0
Northamptonshire					1	0	1	0	1	0	0	0	1	0	0	0	0	0	2	0
Gloucestershire													0	0	0	0	30	8	30	3
Hertfordshire					3	0			11	0			6	0	1	0	50	1	0	0
Hertfordshire													1	0			0	0	6	0
Breconshire	0	0			0	0											0	0	9	0
Oxfordshire	0	0			0	0							0	0			30	30	6	0
Buckinghamshire	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	19
Essex									0	0			0	0			0	0	0	0
Buckinghamshire	2	0			1	0			0	0			0	0			3	0	30	3
Oxfordshire	1	0															30	1	30	0
Oxfordshire	4	0			1	1			1	0			0	0			5	0	0	0
Oxfordshire	0	0	1	0	3	0	1	0	2	0	1	0	1	1	1	0	25	8	25	2
Oxfordshire	0	0			0	0			0	0			0	0			30	2	30	6
Greater London	7	0	5	0	2	0	5	0	3	0	0	0					2	0	17	0
Greater London	1	0	0	0	1	0							0	0			19	0	2	0
Buckinghamshire									0	0	3	0	1	0	1	0	4	0	12	0
Greater London	1	0			0	0							4	0			16	1	8	0
Greater London	0	0															25	9	30	5
Greater London	2	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	30	1	30	2
Hampshire	2	0			1	0			0	0			0	0			30	0	4	0
Wiltshire	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	30	0	0	0
Somerset					1	0	1	0					0	0	1	0	0	0	30	3
Dorset					0	0			0	0			0	0			0	0	0	0
Hampshire	5	1			1	0			1	0			3	0			0	0	0	0
Dorset	5	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	12	0	30	1
Cornwall	0	0			2	0			2	0			0	0			3	0	3	0

Table 5.9: Details of 46 UK locations from which both an air and soil sample were collected, including the county and the number of *A. fumigatus* (A) and azole-resistant *A. fumigatus* (R) colonies grown from each sample. Samples that grew azole-resistant *A. fumigatus* are highlighted in red and locations that grew azole-resistant *A. fumigatus* from both air and soil samples are highlighted in bold.

5.3.12. Growth of *A. fumigatus* and ARAf colonies from air and soil sampled from the same location

There were 46 UK locations from which soil samples were collected that at least one air sample was also collected from (Chapter 4). The numbers of *A. fumigatus* and ARAf colonies grown from these samples are detailed in Table 5.9. Of these locations, 23 (50%) grew ARAf colonies from either an air sample or soil sample, but only 3 (7%) grew ARAf colonies from both.

5.4. Discussion

The primary aim of this chapter was to determine the numbers of *A. fumigatus* and ARAf spores in soils collected from residential gardens in the UK, and to compare these to the numbers of spores in soils collected from elsewhere in the UK by existing studies. Of the 509 soil samples collected by citizen scientists from their gardens, 327 (64%) samples grew a total of 5,174 *A. fumigatus* colonies and 101 (20%) samples grew a total of 736 ARAf colonies. The average CFU/g for the samples that grew *A. fumigatus* was 316, which is higher than the 106 CFU/g in urban soils and 43.5 CFU/g in agricultural soils reported by Bromley *et al.* (2014)¹⁹¹ and considerably higher than the 0-10 CFU/g reported from experimental cropland, commercial wheat fields, woodlands and grass verges by Fraaije *et al.* (2020)⁸⁹. A smaller percentage of the soil samples in this chapter grew *A. fumigatus* colonies compared to those collected by Sewell *et al.* (2019)¹³¹, 64% versus 78% respectively, whereas a larger percentage grew ARAf colonies, 20% versus 6% respectively. The prevalence of ARAf of 14% reported here is greater than the overall ARAf prevalence of 6% found in urban and rural soils in South Wales by Tsitsopoulou *et al.* (2018)¹³⁴ and 9% in experimental cropland and 12% in commercial wheat fields reported by Fraaije *et al.* (2020)⁸⁹. However; it is lower than the 37% prevalence of ARAf in isolates cultured from flower bulbs dipped in azoles reported by Dunne *et al.* (2017)¹³³. Given the prevalence of ARAf detected in this chapter, and by other studies, it is surprising that Van der Torre *et al.* (2020)¹⁹² did not detect any azole-resistance in the 146 *A. fumigatus* isolates cultured from soil samples collected from farms and private gardens across the UK.

The secondary aim of this chapter was to ascertain whether compost, either commercial or homemade, contained more *A. fumigatus* and/or ARAf spores than other garden locations. The main garden locations sampled from were borders ($n = 206$), compost bag ($n = 49$), compost heap ($n = 80$), pot/planter ($n = 115$) and pot/planter plus compost bag ($n = 38$). Compost bag indicated commercial compost, compost heap indicated homemade compost and pot/planter plus compost bag meant the pot/planter had recently been topped up with commercial compost. The greatest concentration of *A. fumigatus* spores was cultured from compost heap samples at 505 CFU/g, followed by compost bag at 451 CFU/g and pot/planter plus compost bag at 321 CFU/g. Soil samples collected from garden locations that did not contain compost had a lower concentration of *A. fumigatus* spores: 254 CFU/g from pot/planter and 204 CFU/g from borders. As citizen scientists were only asked to indicate one garden location from which the soil sample was collected, it is possible that the concentrations of *A. fumigatus* spores from pot/planters and borders were inflated by the recent addition of compost that was not indicated on the questionnaire. Similar observations were made for ARAf spores, with concentrations of 128-289 CFU/g recorded for garden locations involving compost and 98-127 CFU/g for garden locations without compost. These trends were confirmed as statistically significant in association analyses. When compared to soil samples collected from borders, soils collected from compost bags, compost heaps, pot/planters and pot/planters plus compost bag had significantly greater odds of growing *A. fumigatus* and ARAf ($p < 0.01$ for all associations). Furthermore, samples collected from compost bags, compost heaps and pot/planter plus compost bag grew significantly more *A. fumigatus* colonies compared to samples collected from borders. This association was not seen for numbers of ARAf colonies.

There have been a handful of existing studies that have looked for ARAf specifically in compost, in the UK^{131,133,134} and globally^{126,292–294}. Tsitsopoulou *et al.* (2018) sampled compost from agricultural fields ($n = 6$), a horticultural nursery ($n = 1$) and from public areas ($n = 5$) across South Wales that grew four, one and five *A. fumigatus* isolates, respectively; none of which were azole-resistant¹³⁴. Dunne *et al.* (2017) sampled from commercial compost bought from a garden centre in Dublin but do not disclose how many samples were collected or how many *A. fumigatus* were cultured, only that one isolate was azole-resistant¹³³. Sewell *et al.* (2019) sampled from compost ($n = 2$) in London that, combined with samples collected from

flower beds outside a hospital situated ~500m away ($n = 3$), gave a 60% prevalence of ARAf¹³¹. Pugliese *et al.* (2018) collected samples from composting orange peel in Italy and found concentrations of *A. fumigatus* of 8.8×10^3 CFU/g at the start of the process rising to 605.7×10^3 CFU/g by the end of the process, but none of the isolates selected for susceptibility testing ($n = 50$) were azole-resistant²⁹⁴. Santoro *et al.* (2017) sampled from green and brown composts ($n = 11$) in Spain, Hungary and Italy and found concentrations of *A. fumigatus* ranging from 100 to 10.6×10^3 CFU/g, although none of the isolates selected for susceptibility testing ($n = 30$) were azole-resistant²⁹³. Ahangarkani *et al.* (2020) screened 300 compost samples collected in Iran for ARAf and detected 57 isolates with elevated MICs to itraconazole and voriconazole²⁹⁵. Zhang *et al.* (2021) collected a time-series of 114 samples from a plant waste stockpile in the Netherlands and detected $>10^3$ CFU/g in 74% of samples, with the prevalence of ARAf averaging at 50% across all samples²⁹². Schoustra *et al.* (2019) obtained high concentrations of *A. fumigatus* and ARAf from all plant waste sites they sampled: 2.3×10^3 CFU/g and 200 CFU/g, respectively, from household green waste, $>1 \times 10^4$ CFU/g and $1.5-1.8 \times 10^3$ CFU/g from compost heaps in residential gardens, up to 9.4×10^5 CFU/g and up to 2.3×10^5 CFU/g in flower bulb waste and up to 1.9×10^6 CFU/g and up to 8.4×10^4 CFU/g in organic waste from landscaping¹²⁶. While the findings are highly variable with regards to concentrations of *A. fumigatus* and ARAf in composting plant matter and in processed compost, it is clear that both have the potential to contain huge numbers of spores, which warrants further investigation in all countries producing commercial compost including the UK.

The tertiary aim of this chapter was to determine whether environmental variables, aside from the garden location from which the soil sample was collected, influenced spore numbers or azole-resistance in the soils. The variables included in analyses relating to the sample were the date the sample was collected, maximum daily temperature at the sampling location on this date and the land cover classification of the sampling location. The variables relating to the environment around the sampling location were the percentage of arable land in the 2 km buffer surrounding sampling location, the distance between sampling location and the nearest OW/OA composter and the land cover classification of the nearest OW/OA composter. None of these variables affected whether a sample grew *A. fumigatus* or ARAf, or the number of *A. fumigatus* grown from a sample. However, whether the nearest OW/OA

composter was in a rural or urban location affected the number of ARAf colonies grown from a sample, with sampling locations that were closest to an urban composter growing significantly more ARAf than those closest to a rural composter. This finding aligns with the higher prevalence of ARAf in urban locations (13.8%) compared to rural locations (0-1.1%) reported by Sewell *et al.* (2019)¹³¹ and supports their conclusion that horticulture may be a greater driver of azole-resistance than agriculture.

The final aim of this chapter was to characterise the *cyp51A* polymorphisms in ARAf isolates grown from the soil samples. Unfortunately, 93 (13%) of the 736 isolates that grew on tebuconazole at 6 mg/L did not re-grow from short-term storage in the fridge in mould preservation solution, which left 643 (87%) isolates for sequencing of the *cyp51A* promoter and gene regions. Similar to existing UK studies^{131,134,191}, the predominant mutation identified was TR₃₄/L98H (*n* = 542; 74%). Of these isolates, 22 had amino acid substitutions in *cyp51A* in addition to L98H. Interestingly, six isolates with TR₃₄/L98H also contained T289A, I364V and G448S; a combination that has been recently detected in Japan on tulip bulbs imported from the Netherlands²⁹⁶ and in Korea in a patient with IA²⁹⁷. In three isolates TR₃₄ was detected without any accompanying amino acid substitutions and in one isolate TR₆₈/L98H was detected, which was found to be two repeats of the 34 bp insert. The TR₄₆/Y121F/T289A mutation was detected in 16 (2%) isolates and in four additional isolates this was accompanied by S363P, I364V and G448S; a combination detected by Fraaije *et al.* (2020)⁸⁹ in the Netherlands in 2018. Additional polymorphisms detected in this chapter included TR₅₃, which has been previously reported from a patient with multiple-azole-resistant *A. fumigatus* osteomyelitis in the Netherlands¹¹³ and from flower fields in Colombia²⁹⁸, and TR₉₂/Y121F/M172I/T289A/G448S, which is two repeats of the 46 bp insert and has been previously detected in flower bulb waste in the Netherlands¹²⁶. TR₁₃₀/D430G was detected in four isolates; the TR₁₃₀ has been previously detected by a collaborator but is unpublished (personal comms) and the D430G amino acid substitution has been previously detected in a clinical isolate from Japan, associated with voriconazole resistance²⁹⁹. There were 33 (5%) isolates that did not contain any TRs in the promoter region: 27 had no amino acid substitutions in *cyp51A*, five contained I242V and one contained C270R. None of the single *cyp51A* amino acid substitutions detected in this chapter, that occurred alone or alongside

TR₃₄/L98H, are reported in studies summarising *cyp51A* polymorphisms^{108,109,300}, which may suggest these polymorphisms occurred *in situ*.

There were 46 locations from which soil samples were collected for this chapter that were also air sampled in Chapter 3, so these samples were compared for *A. fumigatus* and ARAf growth. There were no locations from which both air and soil samples grew notably high numbers of *A. fumigatus* that might identify them as “hotspots”. At 23 of these locations ARAf was cultured from either an air or a soil sample but only at three locations was ARAf cultured from both. It is likely that this lack of consistency is due to the air and soil sampling rounds each being spaced apart by three months between June 2018 and March 2019. It would be very interesting to repeat the citizen science projects with participants collecting air and soil samples from their garden on the same day, to see if *A. fumigatus* and/or ARAf spore numbers are consistent in both sample types.

The key findings of this chapter were that 64% of soil samples collected from residential gardens in the UK grew *A. fumigatus* colonies, 20% of samples grew ARAf colonies and 14% of all *A. fumigatus* isolates grown were tebuconazole-resistant. While susceptibility testing against medical azoles was not undertaken for these isolates, the majority contained either TR₃₄/L98H or TR₄₆/Y121F/T289A mutations in *cyp51A*, which were associated in Chapter 4 with resistance to three or four of the medical azoles itraconazole, voriconazole, posaconazole and isavuconazole. Fraaije *et al.* (2020)⁸⁹ reported an environmental isolate containing TR₄₆/Y121F/T289A/S363P/I364V/G448S as being resistant to voriconazole and itraconazole, in addition to tebuconazole. Hodiament *et al.* (2009)¹¹³ reported a clinical isolate containing TR₅₃ as being resistant to voriconazole and itraconazole, with reduced susceptibility to posaconazole. This means that individuals in the UK with a garden or gardening occupation are at an elevated risk of being exposed to *A. fumigatus* spores, often in large numbers, that are aerosolised when compost is disturbed; there is a 1 in 6 chance that these spores are azole-resistant. This research raises the question of whether gardening should be listed as a high-risk activity for individuals at risk of contracting aspergillosis. The evidence presented supports the introduction of health warnings on bags of compost and the recommendation for users to wear a mask whilst handling compost. Measures could also be taken by compost producers to sterilise the compost before packaging, thereby killing viable *A. fumigatus* spores and eliminating the immediate hazard it poses to the user. As sterilisation

will be costly, measures to manage the source material for composting could also offer a viable and low-cost solution to mitigating the hazard posed by ARAf.

5.5. Acknowledgements

Thank you to Asmaa Alghamdi of the University of Nottingham and Dr Jianhua Zhang of Wageningen University in the Netherlands for sharing primer details with me for the *cyp51A* promoter and coding regions, respectively. Thank you to Dr Pippa Douglas for providing the locations of composters in England with open windrow or outdoor activity. Thank you to Dr Christopher Uzzell for helping me to download environmental data and the UKCEH Land Cover maps, and for showing me how to use QGIS software to create maps and process these data.

Chapter 6: Is Climate Change a Concern for Azole-Resistant *Aspergillus fumigatus*?

Abstract

As global temperatures rise due to climate change, environmental organisms are expanding their geographical ranges poleward and into higher altitudes resulting in outbreaks of infectious diseases in new locations. These organisms may also develop thermotolerance to survive at mammalian body temperatures, resulting in novel pathogens. Both scenarios apply to fungi, and climate change is expected to expand the range of current fungal pathogens and facilitate emerging fungal infections.

Chapter 4 showed that air samples collected in summer were more likely to grow *Aspergillus fumigatus*, and grew more *A. fumigatus* isolates, than air samples collected in other seasons, which may suggest a role for higher temperatures in *A. fumigatus* survival. In addition, several tebuconazole-resistant isolates cultured from air and soil samples collected in summer lost their resistance after refrigerated storage. In this chapter, I investigated whether these tebuconazole-isolates had lost their *cyp51A* resistance mechanisms whilst being stored at 4°C. I also conducted an 18-week experiment in which *A. fumigatus* isolates cultured from air samples were stored at different temperatures in different storage media to determine whether they lost or gained tebuconazole-resistance.

Sequencing of the *cyp51A* promoter and coding regions revealed that the tebuconazole-resistant isolates cultured from summer air and soil samples had lost their resistance mechanisms during refrigerated storage. However, whole-genome sequencing revealed that the resistant and susceptible versions of the isolates cultured from air samples were not genetically related, which suggests that these isolates were picked from mixed cultures. In the experiment, several *A. fumigatus* isolates that were susceptible to tebuconazole on day 0 gained resistance in subsequent weeks; most whilst being stored at 37°C. *Cyp51A* sequencing showed that some of these isolates had gained the TR₃₄/L98H resistance mechanism, but whole-genome sequencing would need to determine if these isolates were picked from mixed cultures.

It is interesting that long-term storage of a mixed culture at 4°C favoured the survival of the tebuconazole-susceptible *A. fumigatus* isolate over the resistant isolate. Furthermore, storage at 37°C under experimental conditions either induced tebuconazole-resistance in the *A. fumigatus* isolates or favoured the survival of the tebuconazole-resistant *A. fumigatus* isolate over the susceptible isolate in a mixed culture. Further research is required to determine whether temperature affects survival of azole-resistant *A. fumigatus*.

6.1. Introduction

6.1.1. Climate change and infectious diseases

Human activities such as deforestation, farming and the burning of fossil fuels have increased the levels of greenhouse gases in our atmosphere, creating a warming “greenhouse” effect and causing the global mean surface temperature to increase by 0.74°C in the past 100 years³⁰¹. Furthermore, sea levels have risen by 1.8 mm per year since 1961, Arctic sea ice has decreased in coverage by 2.7% per decade, sea surface temperatures are increasing, oceans are becoming more acidic, mountain glaciers are melting and extreme weather events are becoming more frequent and intense³⁰¹. Many studies have discussed the potential implications of climate change for infectious disease (ID) transmission; predominantly with regard to the expanded range of ID hosts and vectors into temperate regions and the contamination of drinking water by waterborne pathogens following extreme rainfall and flooding^{301–306}. Climate variables also have a direct effect on the survival of pathogens: for example, higher ambient temperatures have been linked to increased cases of *Salmonella* and *Campylobacter* infections in Germany³⁰⁷ and to increased cases of cholera in Bangladesh³⁰⁸. There is also an association between increasing temperatures and antimicrobial resistance (AMR), with MacFadden *et al.* (2018) finding that a 10°C increase across regions in the US was associated with increased antibiotic resistance rates of 4.2%, 2.2% and 2.7% for common bacterial pathogens *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, respectively³⁰⁹. Bebbler *et al.* (2013) demonstrated an average poleward shift of 2.7 km/year of crop pests and pathogens - including fungi, bacteria, viruses, viroids, oomycetes, insects and nematodes – since 1960 as a result of warming global temperatures³¹⁰.

Climate change may also increase dispersal of fungal and bacterial pathogens: for example *via* desert dust that is transported great distances through the atmosphere every year and is likely to increase due to storms and desertification³¹¹. This has been observed for the fungus *Coccidioides immitis*, with coccidioidomycosis outbreaks occurring in California following a severe storm in 1977³¹² and an earthquake in 1994³¹³ resulting in wind dispersal of fungal spores in dust clouds.

6.1.2. Climate change and fungal pathogens

6.1.2.1. Plant fungal pathogens

We are already seeing the impact of climate change on several important crops in tropical regions due to the expanding range of fungal pathogens. Mexico is currently struggling to maintain its position as the leading producer of organic coffee due to the rapid spread of coffee leaf rust caused by *Hemileia vastatrix*³¹⁴. In 2012 Mexico saw a 50% loss in national coffee production due to a 1°C temperature rise in the southern mountain regions where coffee is cultivated, which boosted fungal proliferation³¹⁴. Brazil saw high cocoa tree mortality and increased infection rate of the fungal disease witches' broom, caused by *Moniliophthora perniciosa*, during a drought associated with the 2015-16 El Niño event, which caused an 89% reduction in cocoa yield³¹⁵. Species distribution modelling of *Moniliophthora roreri*, the fungus that causes frosty pod rot in cocoa, predicted its expansion across all cocoa-producing countries in South America (Colombia, Venezuela, Peru, Bolivia, Western Brazil) by 2080 and eventual destruction of cocoa plantations due to increased temperature and precipitation caused by climate change³¹⁶.

In temperate regions, winter is a major period of plant pathogen mortality; therefore as climate change increases winter temperatures it is likely that this bottleneck will be removed for many pathogens³¹⁷. There are already several cases of this for fungal pathogens of trees: in a 14-year study in England the fungus responsible for Dutch elm disease, *Ophiostoma novoulmi*, caused greater defoliation in warmer years³¹⁸ and in a 39-year study in Maine *Nectria* species caused greater beech bark cankering following mild winters³¹⁹.

It is difficult to predict whether the same will happen for plant pathogens affecting the major food crops wheat, rice, soybean and potato because the effects are likely to be regional. Furthermore, increasing temperatures will cause a decline in many plant pathogens and

changing CO₂ levels will impact plant biomass³²⁰. Milus *et al.* (2006) found that isolates of *Puccinia striiformis*, which causes stripe rust in wheat, collected in the United States (US) since 2000 were better adapted to warmer temperatures and more aggressive than isolates collected before 2000³²¹. A simulation model used to study the effects of global temperature change on rice leaf blast epidemics, caused by *Magnaporthe grisea*, predicted that elevated temperatures will cause more epidemics in cool subtropics such as Japan and northern China³²². In an analysis of crop pests and pathogens (CPPs), Bebber *et al.* (2014) found that fungi have the narrowest host range of all CPPs but are the most widely dispersed, and also comprise a large proportion of the 50 CPPs that are spreading most rapidly around the globe³²³.

6.1.2.2. Human fungal pathogens

It is hypothesised that climate change will bring about an increase in human fungal infections in two ways: 1) by increasing the geographical range of currently pathogenic species⁷, and 2) by increasing thermotolerance of species and enabling more to survive at human body temperature³²⁴. At present, thermotolerant fungi are more abundant in tropical and subtropical climates and certain pathogens, such as *Paracoccidioides brasiliensis*, *Talaromyces marneffe* and *Coccidioides immitis*, are not found in temperate regions⁷. It is likely that warmer, drier summers have already facilitated the establishment of *C. gattii* in Canada after it infected more than 100 inhabitants of Vancouver Island in 1999³²⁵ and that increasing prevalence of *P. brasiliensis* in Brazil is due to rising humidity levels resulting in greater spore release³²⁶. A 1°C increase in body temperature between 30°C and 42°C excludes 6% of fungal species as potential pathogens³²⁷. As global warming reduces the gap between ambient and human body temperatures, which is currently around 22°C⁷, it is possible that more fungal species will acquire the thermotolerance required to survive at human body temperature. Garcia-Solache and Casadevall (2010) suggest that fungal species that are closely-related to known pathogens, but currently lack thermotolerance, should be characterised as these are the most likely candidates for emerging pathogens. They also warn that the risk from newly emerged fungal pathogens could be magnified by the lack of available antifungal drugs and no licensed vaccines against fungal diseases⁷.

6.1.3. Climate change and antifungal resistance

If climate change does result in an increase in plant and human fungal infections, it will also result in an increase in antifungal use: both in agricultural fungicides and as medicines. Furthermore, unless antifungals with different mechanisms of action or structures are ringfenced for either agricultural or medical use, we are likely to see cross-resistance between environmental and clinical settings, as is currently the case for azole fungicides and aspergillosis infections. Therefore, an indirect effect of climate change would be an increase in antifungal resistance due to increased exposure of target and non-target fungal species to antifungals.

It is possible that climate change may also have a direct effect on inducing antifungal resistance, if thermal adaptation involves gene pathways that are targeted by fungicides. In a US study, isolates of four common chitinolytic fungi collected from an urban heat island grew faster at higher temperatures than the same species collected from a rural area, and the mechanism of thermal adaptation was attributed to increased activity of chitinolytic enzymes³²⁸. A different study found that *Hebeloma* species increase their proteolytic activity to grow at low temperatures³²⁹. Several fungal species, including the major human pathogen *Candida albicans*, have a heat shock response to sudden temperature increases, which induces expression of genes encoding molecular chaperones, proteases and other proteins that protect against cellular damage³³⁰. It is feasible that, in adapting to life at higher ambient temperatures, fungi might upregulate expression of a drug target and thereby evade fungistatic or fungicidal effects.

6.1.4. Hypothesised role of ergosterol in thermal adaptation of *Aspergillus fumigatus*

Sterols are a vital component of all eukaryotic cell membrane and regulate membrane fluidity, permeability and membrane-bound enzymes³³¹. There are three predominant forms of sterols: phytosterols in plants, cholesterol in vertebrates and ergosterol in fungi³³². In fungi, ergosterol is arranged in a monolayer whereas in vertebrates cholesterol is arranged in a bilayer, which is likely due to the absence of a cell wall in vertebrate cells requiring cell membranes to provide greater structural integrity and flexibility³³². The antifungal activity of

azoles is brought about by the inhibition of lanosterol-14 α -demethylase, encoded by *cyp51A* and *cyp51B* genes. Lanosterol-14 α -demethylase is involved in ergosterol biosynthesis in fungi and cholesterol biosynthesis in humans, and antifungal drugs are chosen for having greater binding specificity to the fungal version of the enzyme than to the human version³³³. One of the mechanisms for azole-resistance observed in *Aspergillus fumigatus* involves upregulation of *cyp51A* gene expression, *via* tandem repeats in the gene promoter region, resulting in more lanosterol-14 α -demethylase enzymes being produced than the applied concentration of azoles can bind to and block ergosterol synthesis. All cells must regulate the fluidity of their cell membrane to maintain cell function in response to changing temperatures, which they do by altering the sterol and fatty acid composition of the membrane³³⁴. If fungi increase ergosterol production in response to increasing external temperatures in order to maintain membrane rigidity and regulate permeability, azole-resistance would be a by-product of thermal adaptation. This may have been observed by Zhou *et al.* (2021) who found that ~80% of 233 *A. fumigatus* isolates grown from soils in greenhouses in China were azole-resistant, where temperatures reach up to 45°C³³⁵.

The cholesterol content of plasma membranes has been shown to vary with temperature acclimation, with warm-acclimated rainbow trout³³⁶, crabs³³⁷ and copepods³³⁸ having higher cholesterol content than their cold-acclimated counterparts. In addition, the plasma membranes of freeze-tolerant wood frogs living in Alaska contained less cholesterol than their counterparts in southern Ohio³³⁹. Similar patterns have been found for ergosterol in fungi. Weinstein *et al.* (2000) found that the ergosterol content of *Geomyces pannorum* fell by 50% when it was grown at 5°C compared to 15°C³⁴⁰ and *Saccharomyces cerevisiae* yeast mutants in which ergosterol biosynthesis was blocked were unable to grow at different temperatures³⁴¹. Furthermore, Kamthan *et al.* (2017) found that expression of an ergosterol biosynthesis enzyme in the fission yeast *Schizosaccharomyces pombe* increased its thermotolerance³⁴².

6.1.5. Potential evidence for thermal adaptation of *A. fumigatus* in this thesis

There are two possible examples of thermal adaptation in this thesis: 1) the increased growth of *A. fumigatus* from samples collected in the summer air sampling round when maximum

daily temperatures were at their annual peak compared to the spring and autumn air sampling rounds, and 2) an observed loss of azole-resistance in *A. fumigatus* isolates after long-term storage at 4°C.

The objectives of this chapter were therefore two-fold:

1. to determine whether the *A. fumigatus* isolates cultured from air samples (Chapter 3) and soil samples (Chapter 4) that lost resistance during long-term refrigerated storage were genetically-identical to the *A. fumigatus* isolates from the same tube that were tebuconazole-resistant, and
2. to investigate whether *A. fumigatus* isolates cultured from newly-collected air samples lost or gained tebuconazole-resistance under experimental conditions, which involved storage in three different media and at three different temperatures.

6.2. Methods

6.2.1. Discovery of loss of azole-resistance in *A. fumigatus* isolates grown from air samples

As described in sections 2.3 and 2.4, when the air samplers were received in the post the cover was removed and the sticky side was placed face-down on Sabauroud dextrose agar (SDA) plates containing penicillin (200 mg/L) and streptomycin (400 mg/L). The plates were then incubated for 24 hours at 43°C, the samplers were removed and the plates were incubated for a further 24 hours at 43°C. At this point, colonies that macroscopically resembled *A. fumigatus* were picked from the centre using a sterilised toothpick into a tube containing mould preservation solution (MPS), which was labelled and refrigerated. At a later date, each tube was vortexed and 5 µl of the isolate was pipetted onto a control plate containing SDA agar and dimethyl sulfoxide (DMSO) and onto a drug plate containing SDA agar plus tebuconazole (6 mg/L). The plates were incubated at 37°C for 48 hours and tebuconazole plates exhibiting growth were sealed with parafilm and stored at 4°C.

As described in sections 2.6 and 2.7, the isolates that were able to grow in the presence of tebuconazole were later retrieved from the refrigerator for cryopreservation and DNA extraction, which involved vortexing each tube and pipetting 10 µl of the isolate onto an SDA

plate and 25 cm³ Nunc™ flask containing SDA. At this point the isolates were re-tested for susceptibility to tebuconazole and it was found that several isolates were no longer able to grow in the presence of tebuconazole. When this occurred, the tebuconazole plate on which the isolate had grown originally was retrieved from the refrigerator and the isolate was picked again into a tube containing MPS. Henceforth, all isolates grown directly from air samplers were referred to as primary (1°) isolates and isolates picked from tebuconazole plates were referred to as secondary (2°) isolates.

Both 1° and 2° isolates grown from air samples were taken to the Public Health England (PHE) Mycology Reference Laboratory in Bristol and tested for susceptibility to medical azoles, following Clinical & Laboratory Standards Institute (CLSI) guidelines as detailed in section 2.8.

6.2.2. Discovery of loss of azole-resistance in *A. fumigatus* isolates grown from soil samples

Isolates that had been grown from soil samples and stored at 4°C in MPS were re-tested for susceptibility to tebuconazole at the same time as being cryopreserved and DNA extracted, and several isolates were no longer able to grow in the presence of tebuconazole. As detailed in section 2.5, the first soil samples to be processed were only plated onto SDA plates containing penicillin (200 mg/L) and streptomycin (400 mg/L); therefore all *A. fumigatus* colonies that grew from these samples were individually picked and stored in MPS. As for isolates grown from air samples, 5 µl of each isolate was pipetted onto a control plate containing SDA agar and DMSO and onto a drug plate containing SDA agar plus tebuconazole (6 mg/L) and all tebuconazole plates exhibiting growth were sealed with parafilm and stored at 4°C. This meant that isolates that lost their resistance were picked from tebuconazole plates and labelled as 2° isolates.

When it was decided that this method was unsustainable, because of the high number of *A. fumigatus* colonies being grown from the soil samples (up to 50 colonies), the soil samples were instead replica plating onto an SDA plate containing penicillin and streptomycin and an SDA plate containing penicillin, streptomycin and tebuconazole (6 mg/L). The number of colonies on each plate was recorded and only colonies growing on the plate containing tebuconazole were picked into tubes containing MPS and stored at 4°C. Due to the growth of

bacterial and fungal contaminants on many of the plates, even in the presence of antimicrobials, the decision was made not to store them and they were disposed of after the *A. fumigatus* colonies had been picked. This meant there were no 2° isolates for isolates that had been replica plated.

6.2.3. Sanger sequencing of 1° and 2° isolates

The promoter and coding regions of *cyp51A* were sequenced, as described in section 2.9, for all isolates that lost resistance during storage at 4°C and had both 1° and 2° isolates available.

6.2.4. Whole-genome sequencing of SS18 1° and 2° isolates

High quality genomic DNA (gDNA) was extracted from both the 1° and 2° versions of tebuconazole-resistant isolates collected in the summer air sampling (prefix SS18), purified and sequenced as described in section 2.11.1. The raw WGS reads were processed (section 2.11.2) and RAxML (v8.2.9)¹⁴² was used to calculate the genetic distance between the 1° and 2° SS18 isolates and the 212 UK clinical and environmental isolates from Rhodes *et al.* (2021)²³¹. RAxML does this by generating a distance matrix of isolates, where pairwise distances are between zero and one. A distance of zero indicates closely related isolates and a distance of one indicates unrelated isolates. As this distance represents the distance between isolates on a phylogenetic tree, adding up the distance from the root of the tree to all tips should equal one; therefore, adding or removing isolates would alter the distance. By calculating the number of SNPs separating several pairs of isolates, it is possible to use this distance to calculate the average numbers of SNPs separating each pair of isolates. The isolate pairs were ranked according to the genetic distance between them, from most-related to least-related.

6.2.5. Temperature experiment for loss or gain of azole-resistance in *A. fumigatus* isolates

On 22nd July 2020, 10 air samplers were exposed in several locations around a garden for eight hours. The maximum temperature on this date in Abingdon, Oxfordshire was 21°C. These samples were processed as detailed in section 2.3 and grew 23 colonies that macroscopically resembled *A. fumigatus*, which were all cryopreserved (section 2.6) and DNA extracted

(section 2.7). These isolates were picked into tubes containing MPS (0.2% agar and 0.05% Tween® 20 in dH₂O), Tween (0.05% Tween® 20 in deionised water) and deionised water (diH₂O) and then further split into three tubes per isolate. Each isolate was tested for susceptibility to tebuconazole by pipetting 5 µl from an MPS tube onto a control plate containing SDA agar and DMSO and onto a drug plate containing SDA agar plus tebuconazole (6 mg/L). A tube containing each isolate in MPS, Tween and diH₂O was then placed into the refrigerator at 4°C, room temperature (RT) at 21°C and in an incubator at 37°C. All isolates were re-tested for susceptibility to tebuconazole after 3, 6, 10, 14 and 18 weeks of storage in different media and at different temperatures. Table 6.1 gives an overview of the experimental setup.

Experimental setup		Temperatures		
		4°C	21°C (RT)	37°C
Storage medium	MPS	x	x	x
	Tween	x	x	x
	diH ₂ O	x	x	x
<i>A. fumigatus</i> isolates (x) were tested for susceptibility to 6 mg/L tebuconazole on day 0 and after 3, 6, 10, 14 and 18 weeks of storage.				

Table 6.1: Overview of experimental setup for storage of *A. fumigatus* isolates in three different media and at three different temperatures over 18 weeks to determine whether any isolates lost or gained resistance to tebuconazole at 6 mg/L. RT=room temperature, MPS=mould preservation solution, diH₂O=deionised water.

If the susceptibility of an isolate to tebuconazole changed during the experiment, either by losing or gaining the ability to grow in the presence of tebuconazole, the isolate was cryopreserved, DNA extracted and had the promoter and coding region for *cyp51A* sequenced.

6.3. Results

6.3.1. *A. fumigatus* isolates grown from air samples that lost azole-resistance

There were 14 isolates grown from air samples that lost azole-resistance between their first and second susceptibility test to tebuconazole. The minimum inhibitory concentrations (MICs) for itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ) and *cyp51A* polymorphisms for the 1^o and 2^o isolates are shown in Table 6.2.

Isolate name (SS18-)	1° isolate					2o isolate				
	ITZ	VCZ	PCZ	ISZ	<i>cyp51A</i>	ITZ	VCZ	PCZ	ISZ	<i>cyp51A</i>
005-02-1/4	0.06	0.25	0.03	0.25	WT	16	16	0.5	16	TR ₄₆ /Y121F/T289A
040-01-17/17	0.12	0.25	0.06	0.5	WT	16	2	0.5	2	TR ₃₄ /L98H
056-02-1/6	0.12	0.25	0.03	0.5	WT	16	1	0.5	2	TR ₃₄ /L98H
056-02-4/6	0.12	0.25	0.06	0.25	WT	16	2	0.25	2	TR ₃₄ /L98H
056-02-5/6	0.12	0.5	0.06	0.25	WT	16	2	0.25	2	TR ₃₄ /L98H
068-01-3/6	0.12	0.25	0.06	0.5	WT	16	2	0.25	2	TR ₃₄ /L98H
082-01-5/8	0.06	0.25	0.06	0.25	WT	16	2	0.25	4	TR ₃₄ /L98H
082-01-8/8	0.12	0.25	0.06	0.5	WT	16	2	0.25	4	TR ₃₄ /L98H
190-02-2/2	0.12	0.25	0.06	0.5	WT	16	16	0.5	16	TR ₄₆ /Y121F/T289A
202-02-16/19	0.12	0.25	0.03	0.5	WT	16	4	0.25	2	TR ₃₄ /L98H
204-01-7/16	0.06	0.25	0.03	0.25	WT	16	2	0.25	2	TR ₃₄ /L98H
204-01-12/16	0.06	0.25	0.03	0.25	WT	16	2	0.5	2	TR ₃₄ /L98H
257-01-1/4	0.12	0.25	0.25	0.5	WT	16	2	0.5	2	TR ₃₄ /L98H
381-02-1/2	0.12	0.5	0.03	0.25	D262Y	16	2	0.25	2	TR ₃₄ /L98H/N479S

Table 6.2: The unique identifiers of the 14 tebuconazole-resistant isolates that lost tebuconazole-resistance after refrigeration in mould preservation solution. The minimum inhibitory concentrations (MICs) for itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISZ) and *cyp51A* polymorphisms are given for the primary (1°) and secondary (2°) isolates. All isolates were collected in the summer air sampling round, denoted by the prefix SS18. WT=wild-type. Resistant MICs are highlighted in bold.

All 14 isolates in Table 6.2 were grown from air samples collected in summer. Table 6.3 shows the number of samples collected in each air sampling round that grew azole-resistant *A. fumigatus* (ARAF) and the average time between the first and second susceptibility tests to tebuconazole for these isolates. Figure 6.1 shows the maximum daily temperatures at the sampling locations of samples that grew ARAF in each sampling round.

Air sampling round	Number of samples that grew azole-resistant isolate(s)	Average time between 1 st and 2 nd tebuconazole susceptibility tests (in months)
summer	30	9.0
autumn	14	7.6
winter	12	4.4
spring	17	3.2

Table 6.3: The number of samples that grew azole-resistant isolates and the average time in months between the first and second tebuconazole susceptibility test on isolates, for each air sampling round.

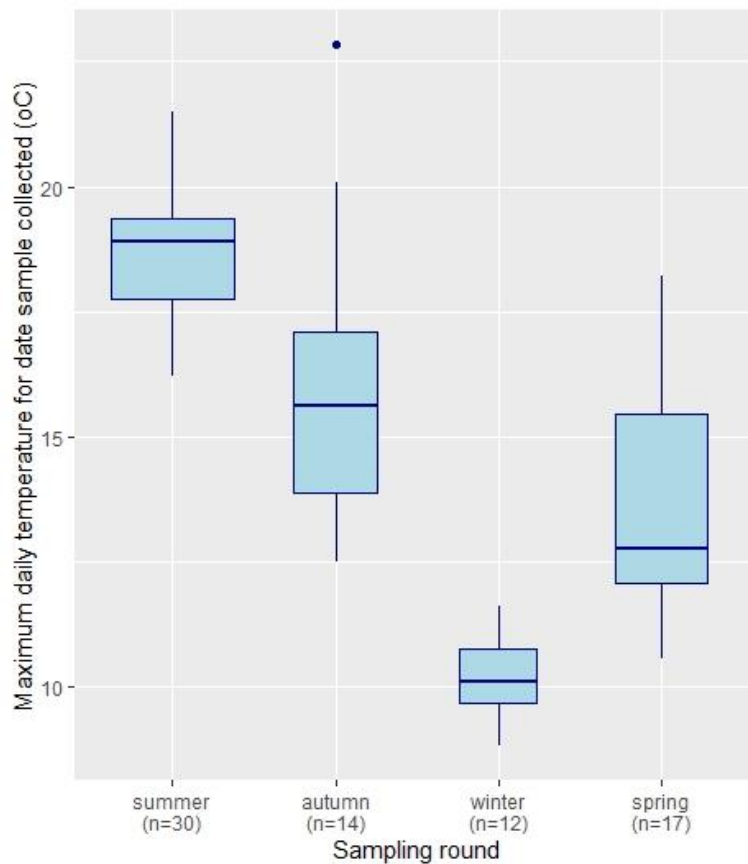


Figure 6.1: Boxplot showing the maximum daily temperatures at the locations that air samples that grew azole-resistant *A. fumigatus* were collected from in each sampling round.

6.3.2.

Whole-genome sequencing of SS18 1° and 2° isolates

Between 13 pairs of 1° and 2° SS18 isolates and 212 UK environmental and clinical isolates, RAxML calculated a total of 28,203 pairwise distances. These distances ranged from 0.003 (approximately 111 SNPs different) to 0.356 (approximately 11,854 SNPs different). Table 6.4 details the genetic distance, SNP differences and relatedness ranking for 13 pairs of 1° and 2° SS18 isolates.

Isolate name	Genetic distance between 1° and 2°	Calculated SNP differences between 1° and 2°	Relatedness ranking (out of 28,203; most to least related)
SS18-040-01-17/17	0.059	1,968	970
SS18-204-01-7/16	0.097	3,244	2,501
SS18-082-01-8/8	0.113	3,772	3,334
SS18-005-02-1/4	0.145	4,820	6,307
SS18-381-02-1/2	0.153	5,105	7,452
SS18-202-02-16/19	0.171	5,703	9,559
SS18-056-02-5/6	0.172	5,724	9,630
SS18-257-01-1/4	0.225	7,493	19,110
SS18-204-01-12/16	0.233	7,745	20,656
SS18-056-02-1/6	0.240	7,982	21,802
SS18-082-01-5/8	0.248	8,255	22,829
SS18-068-01-3/6	0.257	8,563	23,986
SS18-190-02-2/2	0.268	8,933	25,243

Table 6.4: Pairwise genetic distances calculated by RAXML, SNP differences and relatedness rank out of 28,203 for 13 pairs of primary (1°) and secondary (2°) SS18 isolates.

6.3.3. *A. fumigatus* isolates grown from soil samples that lost azole-resistance

There were 28 *A. fumigatus* isolates grown from soil samples that lost their azole-resistance between their first and second susceptibility test to tebuconazole. The isolates and the *cyp51A* polymorphisms in their 1° and 2° isolates are shown in Table 6.5. All of these isolates were picked from SDA plates with antibiotics only into MPS and later tested for susceptibility to tebuconazole; none of the isolates cultured from soil samples directly onto SDA plates containing tebuconazole lost their tebuconazole-resistance during refrigerated storage.

The mean maximum daily temperature on the dates and locations that soil samples that grew azole-resistant isolates ($n = 101$) were collected was 20.1°C, ranging from 12.6°C to 30.5°C. The average time between the first and second tebuconazole susceptibility test, for tebuconazole-resistant isolates that were initially grown on SDA plates without tebuconazole, was 7.7 months.

Isolate name	<i>cyp51A</i> polymorphisms in 1° isolate	<i>cyp51A</i> polymorphisms in 2° isolate
SS19-003-01-8/23	P216R/F219C	TR ₃₄ /L98H
SS19-003-01-14/23	WT	TR ₃₄ /L98H
SS19-007-02-5/6	WT	TR ₃₄ /L98H
SS19-007-02-6/6	TR ₃₄ /L98H	TR ₃₄ /L98H
SS19-009-01-4/25	D262Y	TR ₃₄ /L98H
SS19-009-01-16/25	WT	TR ₃₄ /L98H
SS19-009-01-18/25	WT	TR ₃₄ /L98H/N406M
SS19-009-01-25/25	WT	TR ₃₄ /L98H
SS19-010-01-21/25	WT	TR ₃₄ /L98H
SS19-010-01-22/25	WT	TR ₃₄ /L98H/P443L
SS19-010-01-23/25	I242V	TR ₃₄ /L98H
SS19-010-01-24/25	WT	TR ₃₄ /L98H
SS19-010-01-25/25	WT	TR ₃₄ /L98H
SS19-015-02-2/17	TR ₃₄ /L98H	TR ₃₄ /L98H
SS19-015-02-4/17	TR ₃₄ /L98H	TR ₃₄ /L98H
SS19-015-02-5/17	WT	TR ₃₄ /L98H
SS19-015-02-6/17	WT	TR ₃₄ /L98H
SS19-015-02-15/17	WT	TR ₃₄ /L98H/A460S
SS19-015-02-17/17	WT	TR ₃₄ /L98H
SS19-045-01-9/23	N248K	TR ₃₄ /L98H
SS19-045-01-12/23	I242V	TR ₃₄ /L98H
SS19-045-02-26/31	Failed to sequence	TR ₃₄ /L98H
SS19-054-02-5/18	WT	TR ₃₄ /L98H
SS19-054-02-18/18	WT	TR ₃₄ /L98H
SS19-078-01-5/30	WT	TR ₃₄ /L98H
SS19-078-01-19/30	WT	TR ₃₄ /L98H
SS19-078-01-24/30	WT	TR ₃₄ /L98H
SS19-084-02-12/13	WT	TR ₃₄ /L98H

Table 6.5: The unique identifiers of the 28 tebuconazole-resistant isolates that lost tebuconazole-resistance after refrigeration in mould preservation solution. The *cyp51A* polymorphisms are given for the primary (1°) and secondary (2°) isolates. All isolates were collected in the soil sampling round, denoted by the prefix SS19. WT=wild-type.

6.3.4. *A. fumigatus* isolates that lost or gained azole-resistance during the temperature experiment

Of the 23 *A. fumigatus* isolates grown from the 10 garden air samples, only one isolate (JG7) was resistant to tebuconazole from day 0 and continued to be resistant for the duration of the experiment. There were five isolates JG3, JG8, JG9, JG13 and JG23 that were not resistant to tebuconazole on day 0 but subsequently developed tebuconazole-resistance at a later experimental date. Table 6.6 shows the medium and temperature that each isolate was stored in and the week that the isolate exhibited full (+) or partial (o) growth in the presence of

tebuconazole. Table 6.7 shows the *cyp51A* polymorphisms for isolates that became tebuconazole-resistant, on day 0 and at the time and experimental conditions under which they acquired resistance.

Isolate name	Weeks since experiment started					
	0	3	6	10	14	18
JG3	-	-	o Tween @ 37°C	-	+ MPS @ RT	-
JG8	-	+ MPS @ 4°C	-	-	-	o Tween @ 37°C
JG9	-	+ Tween @ 37°C	-	o MPS @ RT	+ MPS @ RT	-
JG13	-	+ diH ₂ O @ 37°C	+ diH ₂ O @ 37°C	+ diH ₂ O @ 37°C	+ diH ₂ O @ 37°C	+ diH ₂ O @ 37°C
JG23	-	-	-	o Tween @ 37°C	-	o Tween @ 37°C

Table 6.6: Five isolates grown from garden air samples that were susceptible to tebuconazole on day 0 of the experiment but exhibited full (+) or partial (o) growth in the presence of tebuconazole at a later date in the experiment. The storage medium and temperature are detailed for each isolate. RT=room temperature.

Isolate name	<i>cyp51A</i> polymorphisms	
	Week 0	Week(s) when tebuconazole-resistant
JG3	WT	WT (week 6, Tween @ 37°C) WT (week 14, MPS @ RT)
JG7	TR ₃₄ /L98H	ND ^a
JG8	WT	TR ₃₄ /L98H (week 3, MPS @ 4°C) TR ₃₄ /L98H (week 18, Tween @ 37°C)
JG9	WT	TR ₃₄ /L98H (week 3, Tween @ 37°C) TR ₃₄ /L98H (week 14, MPS @ RT)
JG13	WT	TR ₃₄ /L98H (week 3, diH ₂ O @ 37°C)
JG23	No priming	TR ₃₄ /L98H (week 10, Tween @ 37°C) TR ₃₄ /L98H (week 18, Tween @ 37°C)

Table 6.7: *Cyp51A* polymorphisms for isolates at week 0 and at the first week that the isolate grew on tebuconazole for the combination of storage medium and temperature. ^a JG7 was not sequenced again after day 0 as it maintained tebuconazole-resistance throughout the experiment.

6.4. Discussion

The primary objective of this chapter was to determine whether the *A. fumigatus* isolates that lost resistance during long-term refrigerated storage were genetically-identical to the *A. fumigatus* isolates from the same tubes that were tebuconazole-resistant. The *cyp51A* promoter and coding region sequencing showed that all 14 (100%) of the 1^o and 2^o isolates from air samples and 24 of 28 (86%) isolate pairs from soil samples had different genotypes.

Of the 42 total 2° isolates, 36 contained TR₃₄/L98H, four contained TR₃₄/L98H with an additional amino acid substitution and two contained TR₄₆/Y121F/T289A. By comparison, the 42 1° isolates were predominantly wild-type (n = 32; 76%), six had one or two amino acid substitutions in cyp51A, three contained TR₃₄/L98H and one failed to sequence. An indication that the 1° and 2° isolates were not genetically identical was the presence of amino acid substitutions that were present in one isolate and not the other, as these might be expected to remain constant if an isolate lost its tandem repeat resistance mechanism. Due to cost and time restraints, only the SS18 1° and 2° isolates from air samples were WGS. If these isolate pairs had the same genetic background, the genetic distance should be near to zero with few SNP differences; however, this was not the case. The most related 1° and 2° pair was SS18-040-01-17/17, which had 1,968 SNPs different and was ranked 970th of the 28,203 pairs. This means that 969 pairs of SS18 isolates and UK clinical and environmental isolates collected between 2005 and 2017 were more closely related than the most related 1° and 2° SS18 pair. This suggests that mixed cultures were picked from SDA plates into MPS where two spores had landed together on the air samplers, and that one spore was tebuconazole-susceptible and the other was tebuconazole-resistant. It also suggests that the tebuconazole-susceptible isolate outlived the tebuconazole-resistant isolate during long-term refrigerated storage in MPS, which might suggest a fitness cost of azole-resistance at low temperatures. It is likely that the 1° and 2° isolates from soil samples (SS19) were also mixed cultures, which would explain why the “loss-of-resistance” was observed in samples that were picked from SDA plates not containing tebuconazole and not in samples that were replica plated onto SDA plates containing tebuconazole. This is because on the replica plates only the tebuconazole-resistant isolate would grow if two spores landed together on the agar.

It is interesting that these mixed cultures occurred in the summer (SS18) air sampling round but not in the autumn, winter or spring air sampling rounds, for which there are several possible explanations:

1. It is possible that mixed cultures did not occur in subsequent sampling rounds. As air samples collected in summer were found in Chapter 4 to be significantly more likely to grow *A. fumigatus*, and grew more *A. fumigatus* isolates, than samples collected in autumn and spring this may be a valid explanation. However, there was no significant difference in *A. fumigatus* growth from samples collected in summer compared to winter

and the average numbers of isolates grown per air sample in these rounds were 2.8 and 3.1, respectively. Therefore, if the likelihood of picking mixed cultures was associated with the number of isolates grown per air sample, there should have been mixed cultures picked from air samples collected in winter too.

2. The average time between the first and second tebuconazole susceptibility tests performed on an isolate was longer for isolates collected in the summer (SS18) air sampling round than in subsequent rounds: 9.0 months compared to 3.2-7.6 months. If there is a fitness cost to tebuconazole-resistant isolates at lower temperatures that would allow the tebuconazole-susceptible isolates to outlive them, this may only become apparent between 7.6 and 9.0 months. This explanation is unlikely as the “loss-of-resistance” was also observed for soil (SS19) isolates and the average time between the first and second tebuconazole susceptibility tests was 7.7 months.
3. Both the SS18 and SS19 isolates were collected in summer when the differential between ambient outdoor temperature and refrigeration at 4°C was greater than for the autumn, winter and spring air sampling rounds. This could mean that the tebuconazole-resistant isolates circulating at average temperatures of 10.0-16.0°C between autumn and spring were able to adapt to survival at 4°C but those circulating at summer temperatures averaging 18.6-20.1°C could not.
4. Timing of azole agricultural fungicide applications in the UK, and thereby how recent the selection pressure was for spores collected in each sampling round, may have played a role in fitness of tebuconazole-resistant isolates. Perhaps *ARAf* predominates following azole exposure but the fitness cost of resistance makes it less able to survive subsequent environmental exposures, such as temperature and UV fluctuations, as time goes on. Future work should look at the effect of temperature and azole exposure on mixed cultures of wild-type and *ARAf* isolates.

The secondary objective of this chapter was to investigate whether *A. fumigatus* isolates lost or gained tebuconazole-resistance during storage in different media and at different temperatures under experimental conditions. Of the 23 *A. fumigatus* isolates cultured from air samples and included in the experiment, one isolate was tebuconazole-resistant on day 0 and remained so for the 18-week duration under all experimental conditions. *Cyp51A* coding and promoter region sequencing found this isolate to contain TR₃₄/L98H. Five (22%) isolates

were not tebuconazole-resistant on day 0 but were subsequently able to grow in the presence of tebuconazole under at least one experimental condition. Three isolates were found to be wild-type on day 0 but contained TR₃₄/L98H on the weeks they were tebuconazole-resistant, one isolate was wild-type at all the timepoints it was sequenced - despite being tebuconazole-resistant at weeks 6 and 14 - and one isolate failed to sequence on day 0 but contained TR₃₄/L98H on the weeks it was tebuconazole-resistant. Nine experimental conditions resulted from storing isolates in three storage media at three temperatures and in four of these conditions isolates “gained resistance”: MPS at 4°C ($n = 1$), MPS at room temperature ($n = 2$), diH₂O at 37°C ($n = 1$) and Tween at 37°C ($n = 5$). WGS would need to be done on these isolates to determine if they have the same genetic background, as it is possible that the cultures were mixed as for the 1^o and 2^o SS18 isolates. In which case, it is interesting that the higher temperatures in this experiment favoured survival of the tebuconazole-resistant isolates, as opposed to 4°C favouring survival of the tebuconazole-susceptible SS18 and SS19 isolates. It is also possible that the pH of the storage media affected viability of spores, as hyperosmotic stress has been shown to decrease survival of *Saccharomyces cerevisiae* mutants unable to biosynthesise ergosterol³⁴³. In this way, tebuconazole-resistant isolates that contain a tandem repeat in the *cyp51A* promoter region and are thereby producing more ergosterol-synthesising enzyme may have a fitness benefit over wild-type isolates when stored in a medium with a lower pH. The pH of the storage media used in this experiment were not measured but the supplier (Merck, Germany) safety data sheet (SDS) details that a 1% aqueous solution of Tween® 20 has a pH of 5-7. Therefore, it is likely that the MPS and Tween storage media had a lower pH than diH₂O.

A lesson learnt from this chapter is the importance of ensuring that single isolates are picked from environmental samples, placed in storage, and subsequently cryopreserved and DNA extracted. Avoiding picking mixed cultures from soil samples is straight-forward as the sample can be replica-plated onto SDA plates with and without tebuconazole, and isolates picked from the SDA plate containing tebuconazole. This was demonstrated for soil sampling in this thesis as “loss-of-resistance” was not observed in any of the isolates picked from tebuconazole plates. Initial testing showed that replica plating soil samples onto two SDA plates without tebuconazole grew consistently similar numbers of colonies and those plates grew up to 50 colonies. Therefore, the prevalence of ARAf was the number of colonies on the

SDA plate with tebuconazole as a proportion of the number of colonies on the SDA plate without tebuconazole. Avoiding picking mixed cultures from air samples would be less straight-forward as there were fewer isolates cultured from the air samples and they grew from where they landed on the sampler. As the air samplers collected two or three spores on average, it is possible that cutting the sampler in half for replica plating would plate the ARAf spore onto SDA without tebuconazole 50% of the time, thereby underestimating the prevalence of ARAf in air samples. One solution could be to pick each colony twice, from different sides, in the hope of picking pure cultures of each isolate in the mix. It was fortunate that I stored the plates from the first tebuconazole test on the air sampling isolates and SS19 isolates before I switched to replica plating, so I was able to pick the tebuconazole-resistant 2^o isolates and investigate the apparent loss-of-resistance in the 1^o isolates.

The key finding from this chapter is that temperature may confer both a fitness cost and survival advantage on ARAf compared to wild-type isolates, and that these potential effects need to be investigated further. If cold storage has a fitness cost to ARAf, as suggested by SS18 wild-type isolates outliving SS18 ARAf isolates during refrigerated storage, then this could be a valid way of reducing ARAf spore numbers in commercial compost prior to shipping (Chapter 5). On the contrary, if higher temperatures give ARAf a competitive advantage over wild-type isolates we might expect to see a greater prevalence of ARAf in summer months and in future as ambient temperatures increase due to climate change. Both effects could be due to increased ergosterol production by ARAf, as increased ergosterol content would increase survival at higher temperatures when the cell membrane needs to be more fluid and would decrease survival at lower temperatures when the cell membrane needs to be more rigid. More research is needed to find out if ergosterol production is responsible for these potential temperature effects on ARAf survival.

6.5. Acknowledgements

I would like to thank Professor Elizabeth Johnson and Professor Andy Borman of the PHE Mycology Reference Laboratory for letting me conduct CLSI susceptibility testing to medical azoles of the 1^o and 2^o SS18 isolates. Thank you to Asmaa Alghamdi of the University of Nottingham and Dr Jianhua Zhang of Wageningen University in the Netherlands for sharing primer details with me for the *cyp51A* promoter and coding regions, respectively. Thank you

to Samuel Hemmings for extracting DNA from the tebuconazole-resistant SS18 isolates for WGS and to Dr Johanna Rhodes for processing the raw WGS reads and for calculating the genetic distances and SNP differences between isolates that I have included in this chapter.

Chapter 7: General Discussion

Azole-resistant *Aspergillus fumigatus* (ARAF) is a truly “one-health” problem. *A. fumigatus* plays a crucial role in nature by recycling nutrients from decaying organic matter back into the soil, and this is harnessed on a large scale for compost production, which has been recognised by the UK government as a way to replenish nutrient-deficient agricultural soils²⁵². *A. fumigatus* is exposed to azoles in the environment directly, when fungicides are applied to agricultural crops to protect against fungal phytopathogens, and indirectly, *via* residues on the organic matter they are decomposing. Despite not being the target organism, *A. fumigatus* evolves resistance mechanisms to these agricultural azoles in order to survive. When *A. fumigatus* proliferates it releases thousands of microscopic spores into the air that are carried on air currents and inhaled into human lungs, where they can establish infection if not cleared by the innate immune system. It is at this point that *A. fumigatus* becomes the target of medical azoles. However, if the infecting spore has evolved resistance to certain agricultural azoles it will manifest cross-resistance to clinical azoles and salvage therapy may be required.

There is not an aspect of this cycle that can be currently removed or mitigated in order to solve the problem of ARAF. *A. fumigatus* is a globally ubiquitous fungus; it is crucial for nutrient cycling and it would be impossible to eliminate. Compost production is increasing in the UK due to its potential for maintaining soil productivity and for reducing carbon dioxide emissions by diverting waste away from landfill. Azoles are broad-spectrum fungicides found in a third of agricultural sprays²⁸² and are important for protecting crops from disease and spoilage to ensure global food security. The population of individuals in the UK that are susceptible to aspergillosis lung infections is increasing because of the ageing demographic³⁴⁴, improved access to immunosuppressant drugs³⁴⁵ and chemotherapy³⁴⁶ and the COVID-19 pandemic³⁴⁷. As the problem is not going to go away, and indeed looks set to get worse, it is important to understand and monitor the distribution of ARAF and find ways to mitigate the risk of aspergillosis caused by ARAF.

In my thesis I have been assisted by citizen scientists to monitor the levels of *A. fumigatus* and ARAF in air and soils collected from across the UK; to understand whether these are driven by environmental factors such as season, land use or proximity to a composting facility.

7.1. Key findings

The first key finding of my thesis (Chapter 3) was that a citizen science approach to air and soil sampling is valid for mycological surveys. The high number of *A. fumigatus* spores cultured from the air and soil samples ($n = 7,991$) shows that the viability of the spores was not affected by the collection method or storage of the sample at room temperature during postage and prior to processing. Furthermore, the lack of growth of *A. fumigatus* from half of the air samples and one-third of the soil samples suggests that contamination of the samples by individuals who were not necessarily scientists or microbiologists was not an issue. The number of individuals who took part in one or more of the sampling rounds ($n = 787$) also suggests that the sampling method was straightforward and that they were engaged in the research. The organisations The Aspergillosis Trust and National Aspergillosis Centre played an important role in promoting the citizen science projects, and many of the participants shared that they have aspergillosis or have a friend or family member who does. As a result, these organisations and individuals were particularly interested in the results from this thesis. Schools and home-schooling parents were also very engaged in the projects and used the sampling as an opportunity to incorporate microbiology/mycology teaching into their curriculum. It would not have been possible to achieve the objectives of my PhD: 1) to determine the background levels of ARAf spores simultaneously across the UK, and 2) to determine the main routes by which people living in the UK are exposed to *A. fumigatus* and ARAf spores, without the citizen science approach to sample collection. I also believe that these citizen science projects have raised awareness of antifungal drug resistance and aspergillosis amongst the public and within the scientific community.

The second key finding (Chapter 4) was that 5% of *A. fumigatus* spores collected on air samples from across the UK were resistant to the agricultural azole tebuconazole, 4% were resistant to at least one of the medical azoles itraconazole, voriconazole, posaconazole and isavuconazole, and 1% were resistant to all five azoles tested. No apparent spatial clustering of these ARAf spores suggests that individuals everywhere in the UK are at-risk of inhaling ARAf spores, which are associated with treatment failure if they result in aspergillosis infection and increased mortality if the infection progresses to invasive aspergillosis. While there were no significant associations between the growth of ARAf and the environmental variables measured, season was a strong determinant of *A. fumigatus* spore growth and

numbers, with samples collected in summer growing significantly more *A. fumigatus* than samples collected in autumn or spring. This could be due to land use or farming practices throughout the year, or it could be due to thermophilic *A. fumigatus* preferring higher temperatures for sporulation. This may also suggest higher numbers of viable *A. fumigatus* spores as global temperatures rise due to climate change. Another determinant of *A. fumigatus* growth from air samples was proximity of the sampling location to a composting facility with open windrow or outdoor activity (OW/OA). Douglas *et al.* (2017)²³⁹ predicted that airborne *A. fumigatus* levels return to “background levels” within 2 km of a composting facility with OW/OA. According to data downloaded for this thesis, 47,315 of 1,773,498 (3%) UK postcodes are within 2 km of an OW/OA composter. It is not possible to say how many homes and workplaces are based at these postcodes, but the individuals living and working at these postcodes are likely being exposed to “higher than background” levels of spores.

The third key finding (Chapter 5) was that 14% of *A. fumigatus* spores collected in soil samples from residential gardens across the UK were resistant to tebuconazole, and that soil samples containing compost were more likely to grow *A. fumigatus* and ARAf than samples without. While the tebuconazole-resistant isolates from soil samples were not tested for susceptibility to medical azoles, the predominant *cyp51A* polymorphism detected in these isolates was TR₃₄/L98H, which was associated with resistance to itraconazole, voriconazole and isavuconazole in Chapter 4. Gardening is an occupation enjoyed by individuals of all ages in the UK and it is likely that these individuals are exposed to aerosolised *A. fumigatus* and ARAf spores every time they handle compost, either commercial or homemade. A person of any age is able to develop aspergillosis following exposure to a large number of spores^{286–289} but older individuals with weaker immune systems are particularly susceptible, and 72% of 65–74 year olds in the UK report spending their free time gardening²⁸⁵.

It is interesting to note differences between the airborne and soilborne *A. fumigatus* spores that were found to be tebuconazole-resistant in Chapters 4 and 5, respectively. Firstly, 13% of soilborne ARAf isolates failed to regrow after refrigerated storage in mould preservation solution, which was not observed in the airborne ARAf isolates. Secondly, the *cyp51A* polymorphisms observed in the airborne isolates confirmed to be *A. fumigatus* were TR₃₄/L98H (59%), no polymorphism (30%), TR₄₆/Y121F/T289A (6%), or amino acid substitution in *cyp51A* without tandem repeat in the promoter region (4%). By comparison, 26 different

combinations of tandem repeats and amino acid substitutions were recorded in the soilborne isolates confirmed to be *A. fumigatus* including TR₃₄, TR₃₄/L98H (74%), (TR₃₄)²/L98H, TR₄₆/Y121F/T289A (2%), (TR₄₆)²/Y121F/M172I/T289A/G448S, TR₅₃ and TR₁₃₀/D430G as well as no polymorphism (4%). The genotypic variation observed in soilborne isolates but not in airborne isolates may be due to sampling bias due to the greater number of *A. fumigatus* cultured from soil samples compared to air samples: 5,174 isolates from 509 soil samples versus 2,366 isolates from 1,894 air samples. The lack of genotypic variation in airborne isolates may also suggest that *cyp51A* polymorphisms have a fitness cost to spores that makes them unable to remain viable in air, perhaps due to temperature, dehydration or ultraviolet radiation exposure.

The final key finding (Chapter 6) was that long-term storage at 4°C favours survival of tebuconazole-susceptible isolates over tebuconazole-resistant isolates in mixed cultures cultured from air samples collected in summer. This was only observed for isolates collected in the summer air sampling round; possibly because mixed cultures did not occur in subsequent sampling rounds, or because the differential between ambient temperature and refrigeration in autumn, winter and spring was insufficient to assert a fitness cost on the tebuconazole-resistant isolates. Several of the isolates that were cultured from air samples and stored for 18 weeks in different media at different temperature were tebuconazole-susceptible on day 0 and subsequently developed resistance. This change in resistance status predominantly occurred in isolates stored in Tween at 37°C, which could be due to an osmotic effect of Tween or due to temperature. As these isolates were not whole-genome sequenced it is unknown whether the cultures were pure or mixed, but either outcome is interesting. If the cultures were pure, the tebuconazole-susceptible isolate had developed resistance in response to Tween or increased temperature. If the cultures were mixed, Tween or increased temperature favoured survival of the tebuconazole-resistant isolate over the tebuconazole-susceptible isolate. If it is a temperature effect, rather than osmotic effect of Tween, this suggests that lower temperatures favour survival of tebuconazole-susceptible isolates and higher temperatures favour survival of tebuconazole-resistant isolates, which may have future implications with rising temperatures due to climate change.

7.2. Future directions

This thesis has demonstrated that citizen scientist participation is a viable way of collecting environmental samples to monitor for *A. fumigatus* and ARAf across the UK, and that there are members of the public who are motivated to take part in such projects. For the distribution of ARAf in the UK, and the environmental factors driving this distribution, to be fully understood there needs to be a regular and widespread monitoring system in place and I would propose establishing a network of citizen scientists to do so. Such monitoring would benefit from a larger number of citizen scientists collecting samples at set time points throughout the year from locations all over the UK, or a core team of scientists collecting samples daily or weekly from fewer locations. The former would highlight geographical variations in spore numbers in different seasons, whereas the latter would highlight fluctuations in spore numbers throughout the year due to temperature or local farming practices. Either approach would capture longer-term changes in *A. fumigatus* and ARAf spore numbers, which may be expected due to increasing compost production and application to nutrient-depleted agricultural soils, or ambient temperature increases and unpredictable weather due to climate change. Monitoring should also be expanded to measure levels of *A. fumigatus* and ARAf in the UK water system including groundwater³⁴⁸, rivers³⁴⁹ and sewage plants³⁵⁰, as has been done for *A. fumigatus* in other countries.

Environmental sampling is core to the surveillance of many pathogenic microbes that can survive in air, soil and water and it can be challenging for research groups to collect the number and geographical distribution of samples required. This thesis demonstrates that citizen science sample collection is viable for environmental *A. fumigatus* and ARAf surveillance, and there is no reason this approach could not be adapted for other fungal species, bacteria, viruses or toxins. Indeed, the most common contaminants of the air and soil samples collected in this thesis were mucoraceous moulds, which were able to grow at human body temperature and were often tebuconazole-resistant, and have subsequently been the cause of the mucormycosis outbreak in COVID-19 patients in India³⁵¹. The involvement of members of the public also increases awareness of the topic being researched and highlights the importance of research that many individuals otherwise might not understand or acknowledge there is a need for.

Given the likelihood that every individual in the UK is inhaling ARAf spores daily, it is important that individuals who are at the greatest risk of aspergillosis are made aware of this risk and how to mitigate it. This thesis suggests that living or working within 2 km of arable land or an OW/OA composting facility increases an individual's exposure to airborne *A. fumigatus* spores, which high-risk individuals could bear in mind when choosing where to live and work in order to reduce their exposures. Further work should be done on whether wearing face masks reduces inhalation of *A. fumigatus* spores. If they do, high-risk individuals could wear a face mask when working or walking near arable land when compost is being spread or crop residue collected, when handling compost, or when there is landscaping or building work taking place nearby. Finally, experiments should be done to sterilise compost of *A. fumigatus* spores before distribution to understand whether this is viable on a commercial scale; otherwise packaging of commercial compost should be updated to warn users of the risk of inhaling *A. fumigatus* spores when handling it.

Further research needs to be done on the effects of temperature on susceptible and azole-resistant isolates to determine 1) whether isolates (from pure cultures) lose or gain azole-resistance in response to temperature exposure, and 2) whether low or high temperatures confer an advantage for susceptible or azole-resistant isolates in competition experiments. If, as Chapter 6 suggests, low temperatures reduce survival of ARAf, cold storage could be used to reduce the ARAf content of commercial compost. Alternately, if high temperatures favour survival of ARAf it will become even more important to monitor the prevalence and distribution of environmental ARAf in the face of rising ambient temperatures due to climate change.

Finally, widespread azole-resistance in air and soil samples across the UK, coupled with ongoing use of azoles in agricultural fungicides and a growing population of individuals who are susceptible to aspergillosis, highlights our need for new antifungal drugs to be ringfenced for medical use. The impact of azole-resistant aspergillosis on a patient is prolonged or worsening infection while initial azole therapy fails and then either treatment with a higher dose of azoles³⁵², a more toxic antifungal drug or an antifungal drug with lower efficacy³⁵³. Formulation, development and registration of an antifungal drug takes approximately 11 years and costs in the region of 250 million euros³⁵⁴, and the temptation is likely to market the new drug for agricultural use as the UK alone spent £858 million on pesticides in 2019³⁵⁵.

The solution could be an internationalised antifungal development pipeline, with market entry awards and publicly funded antifungal research, as recently proposed for antibiotics in Singer *et al.* (2020)³⁵⁶. Incentives such as these may hasten antifungal drug development and increase global access to new medical antifungals.

References

1. Hawksworth, D. & Gardens, R. B. Fungal Diversity Revisited: 2.2 to 3.8 Million Species. *Microbiol. Spectr.* **5**, 1–17 (2017).
2. Choi, J. & Kim, S. A genome Tree of Life for the Fungi kingdom. *PNAS* **114**, 9391–9396 (2017).
3. Bar-on, Y. M., Phillips, R. & Milo, R. The biomass distribution on Earth. *PNAS* **115**, 6506–6511 (2018).
4. Filip, G. M., Maffei, H. M., Chadwick, K. L. & Max, T. A. Armillaria Root Disease-Caused Tree Mortality following Silvicultural Treatments (Shelterwood or Group Selection) in an Oregon Mixed-Conifer Forest: Insights from a 10-Year Case Study. *West. J. Appl. For.* **25**, 1–8 (2010).
5. Golding, B. Evolution: When was life's first branch point? *Curr. Biol.* **6**, 679–682 (1996).
6. Martin, G. W. Are Fungi Plants? *Mycologia* **47**, 779–792 (1955).
7. Garcia-Solache, M. A. & Casadevall, A. Global warming will bring new fungal diseases for mammals. *MBio* **1**, 1–3 (2010).
8. UN Department of Economic and Social Affairs. *World population prospects 2019. World Population Prospects 2019*. (2019).
9. Bebbber, D. P. & Gurr, S. J. Crop-destroying fungal and oomycete pathogens challenge food security. *Fungal Genet. Biol.* **74**, 62–64 (2015).
10. Fisher, M. C. *et al.* Emerging fungal threats to animal, plant and ecosystem health. *Nature* **484**, 186–194 (2012).
11. FAO. *Food and Agriculture Organization of the United Nations Banana market review: Preliminary results for 2017*. (2017).
12. Dita, M., Barquero, M., Heck, D., Mizubuti, E. S. G. & Staver, C. P. Fusarium wilt of banana: Current knowledge on epidemiology and research needs toward sustainable disease management. *Front. Plant Sci.* **9**, 1–21 (2018).
13. Teixeira, P. J. P. L., Thomazella, D. P. de T. & Pereira, G. A. G. Time for Chocolate: Current Understanding and New Perspectives on Cacao Witches' Broom Disease Research. *PLoS Pathog.* **11**, 1–8 (2015).
14. Aime, M. C. & Phillips-Mora, W. The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycologia* **97**, 1012–1022 (2005).
15. Meinhardt, L. W. *et al.* Moniliophthora perniciosa, the causal agent of witches' broom disease of cacao: What's new from this old foe? *Mol. Plant Pathol.* **9**, 577–588 (2008).
16. Moss, M. O. Fungi, quality and safety issues in fresh fruits and vegetables. *J. Appl. Microbiol.* **104**, 1239–1243 (2008).
17. Cinar, A. & Onbasi, E. *Mycotoxins: The Hidden Danger in Foods*. *IntechOpen* (2012). doi:10.1016/j.colsurfa.2011.12.014
18. Bryden, W. L. Mycotoxins in the food chain: Human health implications. *Asia Pac. J. Clin. Nutr.* **16**, 95–101 (2007).

19. McLeod, G. *et al.* The pathogen causing Dutch elm disease makes host trees attract insect vectors. *Proc. R. Soc. B Biol. Sci.* **272**, 2499–2503 (2005).
20. Erfmeier, A. *et al.* Ash dieback and its impact in near-natural forest remnants – a plant community-based inventory. *Front. Plant Sci.* **10**, 1–12 (2019).
21. Li, G. Q., Liu, F. F., Li, J. Q., Liu, Q. L. & Chen, S. F. Botryosphaeriaceae from eucalyptus plantations and adjacent plants in China. *Persoonia Mol. Phylogeny Evol. Fungi* **40**, 63–95 (2018).
22. Fisher, M. C. *et al.* Threats Posed by the Fungal Kingdom to Humans, Wildlife and Agriculture. *MBio* **11**, 1–17 (2020).
23. Seyedmousavi, S. *et al.* Fungal infections in animals: A patchwork of different situations. *Med. Mycol.* **56**, S165–S187 (2018).
24. Lips, K. R. Overview of chytrid emergence and impacts on amphibians. *Philos. Trans. R. Soc. B Biol. Sci.* **371**, (2016).
25. ACAP. Amphibian Conservation Summit. in *Amphibian Conservation Summit* 17–19 (2005).
26. Voyles, J. *et al.* Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines. *Science (80-.).* **326**, 582–585 (2009).
27. Spitzen-Van Der Sluijs, A. *et al.* Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. *Amphib. Reptil.* **34**, 233–239 (2013).
28. Gargas, A., Trest, M. T., Christensen, M., Volk, T. J. & Blehert, D. S. *Geomyces destructans* sp. nov. associated with Bat white-nose syndrome. *Mycotaxon* **108**, 147–154 (2009).
29. Blehert, D. S. *et al.* Bat white-nose syndrome: An emerging fungal pathogen? *Science (80-.).* **323**, 227 (2009).
30. Puechmaile, S. J. *et al.* White-nose syndrome fungus (*Geomyces destructans*) in Bat, France. *Emerg. Infect. Dis.* **16**, 290–293 (2010).
31. Simmons, N. B. *Mammal species of the World: A Taxonomic and Geographic Reference.* (2005).
32. Kasso, M. & Balakrishnan, M. Ecological and Economic Importance of Bats (Order Chiroptera). *ISRN Biodivers.* **2013**, 1–9 (2013).
33. Fisher, M. C. & Garner, T. W. J. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biol. Rev.* **21**, 2–9 (2007).
34. Bongomin, F., Gago, S., Oladele, R. O. & Denning, D. W. Global and multi-national prevalence of fungal diseases—estimate precision. *J. Fungi* **3**, (2017).
35. Fisher, M. C., Hawkins, N. J., Sanglard, D. & Gurr, S. J. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science (80-.).* **360**, 739–742 (2018).
36. Brown, G. D. *et al.* Hidden killers: Human fungal infections. *Sci. Transl. Med.* **4**, 1–10 (2012).
37. Havlickova, B., Czaika, V. A. & Friedrich, M. Epidemiological trends in skin mycoses worldwide. *Mycoses* **52**, 95 (2009).

38. Sobel, J. D. Vulvovaginal candidosis. *Lancet* **369**, 1961–1971 (2007).
39. Patil, S., Rao, R. S., Majumdar, B. & Anil, S. Clinical appearance of oral Candida infection and therapeutic strategies. *Front. Microbiol.* **6**, 1–10 (2015).
40. Curi, A. L. L., Lazera, M. & Vasconcelos-Santos, D. V. Cryptococcosis. *Intraocular Inflamm.* **30**, 1277–1283 (2016).
41. Rodrigues, M. L. & Albuquerque, P. C. Searching for a change: The need for increased support for public health and research on fungal diseases. *PLoS Negl. Trop. Dis.* **12**, 1–5 (2018).
42. Delaloye, J. & Calandra, T. Invasive candidiasis as a cause of sepsis in the critically ill patient. *Virulence* **5**, 154–162 (2014).
43. Pappas, P. G., Lionakis, M. S., Arendrup, M. C., Ostrosky-Zeichner, L. & Kullberg, B. J. Invasive candidiasis. *Nat. Rev. Dis. Prim.* **4**, 1–20 (2018).
44. Jeffery-Smith, A. *et al.* Candida auris: A review of the literature. *Clin. Microbiol. Rev.* **31**, 1–18 (2018).
45. Chowdhary, A., Sharma, C. & Meis, J. F. Candida auris: A rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. *PLoS Pathog.* **13**, 1–10 (2017).
46. Troung, J. & Ashurst, J. V. *Pneumocystis (Carinii) Jiroveci Pneumonia*. (2020).
47. Edman, J. C. *et al.* Ribosomal RNA sequence shows Pneumocystis carinii to be a member of the Fungi. *Nature* **334**, 519–522 (1988).
48. Davis, J. L. & Huang, L. Pneumocystis Pneumonia. *N. Engl. J. Med.* **350**, 2487–98 (2004).
49. Brakhage, A. A. & Langfelder, K. Menacing Mold: The Molecular Biology of Aspergillus fumigatus. *Annu. Rev. Microbiol.* **56**, 433–455 (2002).
50. Boddy, L. *Fungi, Ecosystems, and Global Change. The Fungi* (Elsevier Ltd., 2016). doi:10.1016/B978-0-12-382034-1.00011-6
51. Latgé, J. P. Aspergillus fumigatus and Aspergillosis. *Clin. Microbiol. Rev.* **12**, 310–350 (1999).
52. Klich, M. A. Biogeography of Aspergillus species in soil and litter. *Mycologia* **94**, 21–27 (2002).
53. Kwon-Chung, K. J. & Sugui, J. A. Aspergillus fumigatus- What Makes the Species a Ubiquitous Human Fungal Pathogen? *PLoS Pathog.* **9**, 1–4 (2013).
54. Ene, I. V. & Bennett, R. J. The cryptic sexual strategies of human fungal pathogens. *Nat. Rev. Microbiol.* **12**, 239–251 (2014).
55. O’Gorman, C. M., Fuller, H. T. & Dyer, P. S. Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. *Nature* **457**, 471–474 (2009).
56. Zhang, J. *et al.* Asexual sporulation facilitates adaptation: The emergence of azole resistance in Aspergillus fumigatus. *Evolution (N. Y.)* **69**, 2573–2586 (2015).
57. O’Gorman, C. M. Airborne Aspergillus fumigatus conidia: a risk factor for aspergillosis. *Fungal Biol. Rev.* **25**, 151–157 (2011).
58. Herr, C. E. W. *et al.* Effects of bioaerosol polluted outdoor air on airways of residents: A cross sectional study. *Occup. Environ. Med.* **60**, 336–342 (2003).
59. Nadal, M., Inza, I., Schuhmacher, M., Figueras, M. J. & Domingo, J. L. Health risks of the occupational exposure to microbiological and chemical pollutants in a municipal waste

- organic fraction treatment plant. *Int. J. Hyg. Environ. Health* **212**, 661–669 (2009).
60. Nikaeen, M., Mirhendi, H., Hatamzadeh, M. & Ghorbani, E. Bioaerosol emissions from composting facilities as a potential health risk for composting workers. *Recent Adv. Environ. Ecosyst. Dev.* 27–29 (2009).
 61. Stagg, S., Bowry, A., Kelsey, A. & Crook, B. *Bioaerosol emissions from waste composting and the potential for workers' exposure. Health and Safety Executive* (2010).
 62. Taha, M. P. M., Pollard, S. J. T., Sarkar, U. & Longhurst, P. Estimating fugitive bioaerosol releases from static compost windrows: Feasibility of a portable wind tunnel approach. *Waste Manag.* **25**, 445–450 (2005).
 63. Taha, M. P. M., Drew, G. H., Longhurst, P. J., Smith, R. & Pollard, S. J. T. Bioaerosol releases from compost facilities: Evaluating passive and active source terms at a green waste facility for improved risk assessments. *Atmos. Environ.* **40**, 1159–1169 (2006).
 64. Wheeler, P. A., Stewart, I., Dumitrean, P. & Donovan, B. *Health Effects of Composting. A study of three compost sites and review of past data. R&D Technical Report P1-315/TR. Environment Agency, UK.* (2001).
 65. Alshareef, F. & Robson, G. D. Prevalence, persistence, and phenotypic variation of *Aspergillus fumigatus* in the outdoor environment in Manchester, UK, over a 2-year period. *Med. Mycol.* **52**, 367–375 (2014).
 66. Mullins, J., Harvey, R. & Seaton, A. Sources and incidence of airborne *Aspergillus fumigatus* (Fres). *Clin. Exp. Allergy* **6**, 209–217 (1976).
 67. Millner, P. D., Marsh, P. B., Snowden, R. B. & Parr, J. F. Occurrence of *Aspergillus fumigatus* during composting of sewage sludge. *Appl. Environ. Microbiol.* **34**, 765–772 (1977).
 68. Beffa, T. *et al.* Mycological control and surveillance of biological waste and compost. *Med. Mycol.* **36**, 137–45 (1998).
 69. Wery, N. Bioaerosols from composting facilities - a review. *Front. Cell. Infect. Microbiol.* **4**, 1–9 (2014).
 70. Rashid, M. I. *et al.* Bacteria and fungi can contribute to nutrients bioavailability and aggregate formation in degraded soils. *Microbiol. Res.* **183**, 26–41 (2016).
 71. Tarafdar, J. C., Rao, A. V. & Bala, K. Production of phosphatases by fungi isolated from desert soils. *Folia Microbiol. (Praha)*. **33**, 453–457 (1988).
 72. Tarafdar, J. C. & Marschner, H. Dual inoculation with *Aspergillus fumigatus* and *Glomus mosseae* enhances biomass production and nutrient uptake in wheat (*Triticum aestivum* L.) supplied with organic phosphorus as Na-phytate. *Plant Soil* **173**, 97–102 (1995).
 73. Hohl, T. M. & Feldmesser, M. *Aspergillus fumigatus*: Principles of pathogenesis and host defense. *Eukaryot. Cell* **6**, 1953–1963 (2007).
 74. Margalit, A. & Kavanagh, K. The innate immune response to *Aspergillus fumigatus* at the alveolar surface. *FEMS Microbiol. Rev.* **39**, 670–687 (2015).
 75. Moss, R. B. Treatment options in severe fungal asthma and allergic bronchopulmonary aspergillosis. *Eur. Respir. J.* **43**, 1487–1500 (2014).
 76. Burgel, P. R., Paugam, A., Hubert, D. & Martin, C. *Aspergillus fumigatus* in the cystic fibrosis lung: Pros and cons of azole therapy. *Infect. Drug Resist.* **9**, 229–238 (2016).

77. Richardson, M., Bowyer, P. & Sabino, R. The human lung and *Aspergillus*: You are what you breathe in? *Med. Mycol.* **57**, 145–154 (2019).
78. Maghrabi, F. & Denning, D. W. The Management of Chronic Pulmonary Aspergillosis: The UK National Aspergillosis Centre Approach. *Curr. Fungal Infect. Rep.* **11**, 242–251 (2017).
79. Dagenais, T. R. T. & Keller, N. P. Pathogenesis of *Aspergillus fumigatus* in invasive aspergillosis. *Clin. Microbiol. Rev.* **22**, 447–465 (2009).
80. Van De Veerdonk, F. L. *et al.* Influenza-associated aspergillosis in critically ill patients. *Am. J. Respir. Crit. Care Med.* **196**, 524–527 (2017).
81. De Foer, C., Fossion, E. & Vaillant, J. M. Sinus aspergillosis. *J. Cranio-Maxillofacial Surg.* **18**, 33–40 (1990).
82. Armstrong-James, D. *et al.* Confronting and mitigating the risk of COVID-19 associated pulmonary aspergillosis. *Eur. Respir. J.* **56**, (2020).
83. Pegorie, M., Denning, D. W. & Welfare, W. Estimating the burden of invasive and serious fungal disease in the United Kingdom. *J. Infect.* **74**, 60–71 (2017).
84. Borman, A. M. *et al.* COVID-19-Associated Invasive Aspergillosis: Data from the UK National Mycology Reference Laboratory. *J. Clin. Microbiol.* **59**, 1–12 (2021).
85. Balkovec, J. M. *et al.* Discovery and development of first in class antifungal caspofungin (CANCIDAS®)—A case study. *Nat. Prod. Rep.* **31**, 1–142 (2014).
86. Maertens, J. A. History of the development of azole derivatives. *Clin. Microbiol. Infect.* **10**, 1–10 (2004).
87. Denning, D. W. *et al.* Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **41**, 1364–1368 (1997).
88. Peyton, L. R., Gallagher, S. & Hashemzadeh, M. Triazole antifungals: A review. *Drugs of Today* **51**, 705–718 (2015).
89. Fraaije, B., Atkins, S., Hanley, S., Macdonald, A. & Lucas, J. The Multi-Fungicide Resistance Status of *Aspergillus fumigatus* Populations in Arable Soils and the Wider European Environment. *Front. Microbiol.* **11**, 1–17 (2020).
90. Andrews, J. M. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* **48**, 5–16 (2001).
91. Marichal, P. & Vanden Bossche, H. Mechanisms of resistance to azole antifungals. *Acta biochimica Polonica* **42**, 509–516 (1995).
92. Chryssanthou, E. In vitro susceptibility of respiratory isolates of *Aspergillus* species to itraconazole and amphotericin B. acquired resistance to itraconazole. *Scand. J. Infect. Dis.* **29**, 509–512 (1997).
93. Dannaoui, E. *et al.* Acquired itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* **47**, 333–340 (2001).
94. Snelders, E. *et al.* Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med.* **5**, 1629–1637 (2008).
95. Howard, S. J. *et al.* Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* **15**, 1068–1076 (2009).

96. Chen, Y. *et al.* High azole resistance in *Aspergillus fumigatus* isolates from strawberry fields, China, 2018. *Emerg. Infect. Dis.* **26**, (2020).
97. Manavathu, E. K., Cutright, J. L., Loebenberg, D. & Chandrasekar, P. H. A comparative study of the in vitro susceptibilities of clinical and laboratory-selected resistant isolates of *Aspergillus* spp. to amphotericin B, itraconazole, voriconazole and posaconazole (SCH 56592). *J. Antimicrob. Chemother.* **46**, 229–234 (2000).
98. Warris, A., Weemaes, C. M. & Verweij, P. E. Multidrug Resistance in *Aspergillus fumigatus*. *N. Engl. J. Med.* **347**, (2002).
99. Howard, S. J. *et al.* Multi-azole resistance in *Aspergillus fumigatus*. *Int. J. Antimicrob. Agents* **28**, 450–453 (2006).
100. Meis, J. F., Chowdhary, A., Rhodes, J. L., Fisher, M. C. & Verweij, P. E. Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus*. *Philos. Trans. B* **371**, 1–10 (2016).
101. Bueid, A. *et al.* Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J. Antimicrob. Chemother.* **65**, 2116–2118 (2010).
102. van der Linden, J. W. M. *et al.* Clinical implications of azole resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009. *Emerg. Infect. Dis.* **17**, 1846–1854 (2011).
103. Bitar, D. *et al.* Population-Based Analysis of Invasive Fungal Infections, France, 2001–2010. *Emerg. Infect. Dis.* **20**, 1149–1155 (2014).
104. Morton, V. & Staub, T. A Short History of Fungicides. *APSnet Featur. Artic.* 1–12 (2008). doi:10.1094/apsnetfeature-2008-0308
105. Kleinkauf, N. *et al.* *European Centre for Disease Prevention and Control: Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in Aspergillus species.* (2013).
106. Hof, H. Is there a serious risk of resistance development to azoles among fungi due to the widespread use and long-term application of azole antifungals in medicine? *Drug Resist. Updat.* **11**, 25–31 (2008).
107. Di Tullio, E., Camanzi, L., Fontolan, F., Volpato, C. & Zucconi, S. *The assessment of the economic importance of azoles in European agriculture: wheat case study.* (2012). doi:10.13140/RG.2.2.10636.31367
108. van der Torre, M. H., Novak-Frazer, L. & Rautemaa-Richardson, R. Detecting azole-antifungal resistance in *Aspergillus fumigatus* by pyrosequencing. *J. Fungi* **6**, 1–15 (2020).
109. Rybak, J. M., Fortwendel, J. R. & Rogers, P. D. Emerging threat of triazole-resistant *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* **74**, 835–842 (2019).
110. Mellado, E. *et al.* A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrob. Agents Chemother.* **51**, 1897–1904 (2007).
111. Wiederhold, N. P. *et al.* First detection of TR34 L98H and TR46 Y121F T289A Cyp51 mutations in *Aspergillus fumigatus* isolates in the United States. *J. Clin. Microbiol.* **54**, 168–171 (2016).
112. Van Der Linden, J. W. M. *et al.* Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin. Infect. Dis.* **57**, 513–520 (2013).

113. Hodiament, C. J. *et al.* Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. *Med. Mycol.* **47**, 217–220 (2009).
114. Hare, R. K. *et al.* In vivo selection of a unique tandem repeat mediated azole resistance mechanism (TR120) in *Aspergillus fumigatus* cyp51A, Denmark. *Emerg. Infect. Dis.* **25**, 577–580 (2019).
115. Verweij, P. E., Mellado, E. & Melchers, W. J. G. Multiple-Triazole–Resistant Aspergillosis. *N. Engl. J. Med.* 1481–1483 (2007).
116. Lemaire, B. *et al.* Hospitalized Patient as Source of *Aspergillus fumigatus*, 2015. *Emerg. Infect. Dis.* **24**, 1524–1527 (2018).
117. Warris, A. *et al.* Molecular epidemiology of *Aspergillus fumigatus* isolates recovered from water, air, and patients shows two clusters of genetically distinct strains. *J. Clin. Microbiol.* **41**, 4101–4106 (2003).
118. Mortensen, K. L. *et al.* Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob. Agents Chemother.* **54**, 4545–4549 (2010).
119. Rocchi, S. *et al.* Determination of azole fungal residues in soils and detection of *Aspergillus fumigatus*-resistant strains in market gardens of Eastern France. *Environ. Sci. Pollut. Res.* **25**, 1–9 (2018).
120. Bader, O. *et al.* Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. *Antimicrob. Agents Chemother.* **59**, 4356–4359 (2015).
121. Chowdhary, A., Sharma, C., Kathuria, S., Hagen, F. & Meis, J. F. Azole-resistant *Aspergillus fumigatus* with the environmental TR46/Y121F/T289A mutation in India. *J. Antimicrob. Chemother.* **69**, 555–557 (2014).
122. Prigitano, A. *et al.* Azole-resistant *Aspergillus fumigatus* in the environment of Northern Italy, May 2011 to June 2012. *Eurosurveillance* **19**, 1–7 (2014).
123. Kemoi, E. K., Nyerere, A. & Bii, C. C. Triazole-resistant *Aspergillus fumigatus* from fungicide-experienced soils in Naivasha subcounty and Nairobi county, Kenya. *Int. J. Microbiol.* (2018). doi:10.1155/2018/7147938
124. Meneau, I. & Sanglard, D. Azole and fungicide resistance in clinical and environmental *Aspergillus fumigatus* isolates. *Med. Mycol.* **43**, 307–311 (2005).
125. Chowdhary, A. *et al.* Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania. *J. Antimicrob. Chemother.* **69**, 2979–2983 (2014).
126. Schoustra, S. E. *et al.* Environmental hotspots for azole resistance selection of *Aspergillus fumigatus*, the Netherlands. *Emerg. Infect. Dis.* **25**, (2019).
127. Camps, S. M. T. *et al.* Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: a case study and review of the literature. *Antimicrob. Agents Chemother.* **56**, 10–16 (2012).
128. Snelders, E. *et al.* Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One* **7**, (2012).
129. Gilbert, E. J., Adrian, K., Karnon, J. D., Swan, J. R. & Crook, B. Preliminary Results of Monitoring the Release of Bioaerosols from Composting Facilities in the UK: Interpretation,

- Modelling and Appraisal of Mitigation Measures. *Biocycle* (2002).
130. Environment Agency. *M9: Environmental monitoring of bioaerosols at regulated facilities*. (2018).
 131. Sewell, T. R. *et al.* Elevated Prevalence of Azole-Resistant *Aspergillus fumigatus* in Urban versus Rural Environments in the United Kingdom. *Antimicrob. Agents Chemother.* **63**, 1–8 (2019).
 132. Bromley, M. J. *et al.* Occurrence of azole-resistant species of *Aspergillus* in the UK environment. *J. Glob. Antimicrob. Resist.* **2**, 276–279 (2014).
 133. Dunne, K., Hagen, F., Pomeroy, N., Meis, J. F. & Rogers, T. R. Intercountry Transfer of Triazole-Resistant *Aspergillus fumigatus* on Plant Bulbs. *Clin. Infect. Dis.* **65**, (2017).
 134. Tsitsopoulou, A. *et al.* Determination of the prevalence of triazole resistance in environmental *Aspergillus fumigatus* strains isolated in South Wales, UK. *Front. Microbiol.* **9**, 1–8 (2018).
 135. Zhang, J. *et al.* A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *MBio* **8**, (2017).
 136. Campbell, C. K., Johnson, E. M. & Warnock, D. W. *Identification of Pathogenic Fungi 2nd Edition*. (2013).
 137. Nierman, W. C. *et al.* Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438**, 1151–1156 (2005).
 138. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).
 139. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
 140. McKenna, A. *et al.* The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* **20**, 1297–1303 (2010).
 141. Smit, A., Hubley, R. & Green, P. RepeatMasker Open-4.0. Available at: <http://www.repeatmasker.org>. (Accessed: 12th July 2021)
 142. Stamatakis, A. RAXML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690 (2006).
 143. Met Office. HadUK-Grid datasets. Available at: <https://www.metoffice.gov.uk/research/climate/maps-and-data/data/haduk-grid/datasets>. (Accessed: 27th January 2021)
 144. QGIS. Available at: <https://www.qgis.org/en/site/>. (Accessed: 27th January 2021)
 145. UKCEH: Land Cover Map 2019. Available at: <https://catalogue.ceh.ac.uk/documents/31f4887a-1691-4848-b07c-61cdc468ace7>. (Accessed: 27th January 2021)
 146. UK Centre for Ecology and Hydrology. *The UKCEH Land Cover Maps for 2017, 2018 and 2019*. (2020).
 147. Scottish Environment Protection Agency (SEPA): Waste Sites. (2019). Available at: <https://www.sepa.org.uk/data-visualisation/waste-sites-and-capacity-tool/>. (Accessed: 4th May 2021)

148. Natural Resources Wales: Environmental Permitting Regulations – Waste Sites. (2021). Available at: <http://lle.gov.wales/catalogue/item/EnvironmentalPermittingRegulationsWasteSites/?lang=en>. (Accessed: 4th May 2021)
149. Northern Ireland Environment Agency: Waste Licenses Register. Available at: <https://apps.d.aera-ni.gov.uk/wastelicences/>. (Accessed: 4th May 2021)
150. Team, R. C. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (2020). Available at: <https://www.r-project.org/>.
151. Sparks, T. H. & Carey, P. D. The Responses of Species to Climate Over Two Centuries: An Analysis of the Marsham Phenological Record. *Br. Ecol. Soc.* **83**, 321–329 (1995).
152. Collinson, N. & Sparks, T. Phenology—nature’s calendar: an overview of results from the UK Phenology Network. *Arboric. J.* **30**, 271–278 (2008).
153. Amano, T., Smithers, R. J., Sparks, T. H. & Sutherland, W. J. A 250-year index of first flowering dates and its response to temperature changes. *Proc. R. Soc. B Biol. Sci.* **277**, 2451–2457 (2010).
154. RSPB (2021) Big Garden Birdwatch. Available at: <https://www.rspb.org.uk/get-involved/activities/birdwatch/>. (Accessed: 15th January 2021)
155. Butterfly Conservation (2020) Big Butterfly Count. Available at: <https://bigbutterflycount.butterfly-conservation.org/>. (Accessed: 15th January 2021)
156. Moors for the Future: The Big Moss Map. Available at: <https://www.moorsforthefuture.org.uk/get-involved/citizen-science-surveys-and-sightings2/sphagnum-survey>. (Accessed: 15th January 2021)
157. Moors for the Future: Hare survey. Available at: <https://www.moorsforthefuture.org.uk/get-involved/citizen-science-surveys-and-sightings2/hare-survey>. (Accessed: 15th January 2021)
158. Goodenough, A. E., Little, N., Carpenter, W. S. & Hart, A. G. Birds of a feather flock together : Insights into starling murmuration behaviour revealed using citizen science. *PLoS One* **12**, 1–18 (2017).
159. Sparks, T. H., Atkinson, S., Lewthwaite, K., Dhap, R. & Moran, N. J. Can bird abundance declines be detected by citizen science programmes? A case study using Common Cuckoo *Cuculus canorus*. *Avian Biol. Res.* **10**, 241–245 (2017).
160. Newson, S. E. *et al.* Long-term changes in the migration phenology of UK breeding birds detected by large-scale citizen science recording schemes. *Int. J. Avian Sci.* **156**, 481–495 (2016).
161. Hof, A. R. & Bright, P. W. Quantifying the long-term decline of the West European hedgehog in England by subsampling citizen-science datasets. *Eur. J. Wildl. Res.* 407–413 (2016). doi:10.1007/s10344-016-1013-1
162. Wilson, J. F. *et al.* A role for artificial night-time lighting in long-term changes in populations of 100 widespread macro-moths in UK and Ireland: a citizen-science study. *J. Insect Conserv.* **22**, 189–196 (2018).
163. Sumner, S., Bevan, P., Hart, A. G. & Isaac, N. J. B. Mapping species distributions in 2 weeks using citizen science. *Insect Conserv. Divers.* **12**, 382–388 (2019).
164. Lye, G. C., Osborne, J. L., Park, K. J. & Goulson, D. Using citizen science to monitor *Bombus*

- populations in the UK: nesting ecology and relative abundance in the urban environment. *J. Insect Conserv.* 697–707 (2012). doi:10.1007/s10841-011-9450-3
165. Gardiner, M. M. *et al.* Lessons from lady beetles: accuracy of monitoring data from US and UK citizen-science programs. *Front. Ecol.* (2012). doi:10.1890/110185
 166. Pocock, M. J. O. & Evans, D. M. The Success of the Horse-Chestnut Leaf-Miner, *Cameraria ohridella*, in the UK Revealed with Hypothesis-Led Citizen Science. *PLoS One* **9**, 1–9 (2014).
 167. Ransom Hardison, D. *et al.* Habscope: A tool for use by citizen scientists to facilitate early warning of respiratory irritation caused by toxic blooms of *Karenia brevis*. *PLoS One* **14**, 1–17 (2019).
 168. Lawson, B. *et al.* Emergence of a Novel Avian Pox Disease in British Tit Species. *PLoS One* **7**, (2012).
 169. Robinson, R. A. *et al.* Emerging Infectious Disease Leads to Rapid Population Declines of Common British Birds. *PLoS One* **5**, (2010).
 170. Brown, N., Bosch, F. Van Den, Parnell, S., Denman, S. & Brown, N. Integrating regulatory surveys and citizen science to map outbreaks of forest diseases: acute oak decline in England and Wales. *Proc. R. Soc. B* (2017).
 171. Snik, F. *et al.* Mapping atmospheric aerosols with a citizen science network of smartphone spectropolarimeters. *Geophys. Res. Lett.* **41**, 7351–7358 (2014).
 172. Tregidgo, D. J., West, S. E. & Ashmore, M. R. Can citizen science produce good science? Testing the OPAL Air Survey methodology, using lichens as indicators of nitrogenous pollution. *Environ. Pollut.* **182**, 448–451 (2013).
 173. McDonald, D. *et al.* American Gut: an Open Platform for Citizen Science Microbiome Research. *mSystems* **3**, 1–28 (2018).
 174. Dunn, R. R., Fierer, N., Henley, J. B., Leff, J. W. & Menninger, H. L. Home Life: Factors Structuring the Bacterial Diversity Found within and between Homes. *PLoS One* **8**, (2013).
 175. Hulcr, J. *et al.* A Jungle in There: Bacteria in Belly Buttons are Highly Diverse, but Predictable. *PLoS One* **7**, (2012).
 176. Urban, J. *et al.* The effect of habitual and experimental antiperspirant and deodorant product use on the armpit microbiome. *PeerJ* **2016**, 1–20 (2016).
 177. LSTM: Swab & Send. Available at: <https://www.lstmed.ac.uk/public-engagement/swab-send>. (Accessed: 15th January 2021)
 178. Bordenstein, S. R. Discover the Microbes Within!: The Wolbachia Project. *Focus Microbiol. Educ.* **14**, 4–5 (2007).
 179. Fungi of Great Britain and Ireland: The Lost and Found Fungi Project. Available at: <http://fungi.myspecies.info/content/lost-and-found-fungi-project>. (Accessed: 15th January 2021)
 180. Österreichische Mykologische Gesellschaft: Pilzfinder. Available at: <https://www.univie.ac.at/oemykges/pilzfinder-at/>. (Accessed: 15th January 2021)
 181. Reese, A. T., Madden, A. A., Joossens, M., Lacaze, G. & Dunn, R. R. Influences of Ingredients and Bakers on the Bacteria and Fungi in Sourdough Starters and Bread. *mSphere* **5**, 1–15 (2020).

182. Spletni Portal Invazivke. Available at: <https://www.invazivke.si/>. (Accessed: 15th January 2021)
183. Living Ash Project. Available at: <https://livingashproject.org.uk/>. (Accessed: 15th January 2021)
184. Pepys, J. Hypersensitivity diseases of the lungs due to fungi and organic dusts. *Monogr Allergy* **4**, 1–147 (1969).
185. Sánchez-Monedero, M. A. & Stentiford, E. I. Generation and dispersion of airborne microorganisms from composting facilities. *Process Saf. Environ. Prot.* **81**, 166–170 (2003).
186. Hall, S., Gibson, H., Lambert, A. & Hill, D. Bioaerosol Monitoring - A Practical Evaluation of Methodology. in *16th European Biosolids & Organic Resources Conference* (2011).
187. Deacon, L. *et al.* Endotoxin emissions from commercial composting activities. *Environ. Heal. A Glob. Access Sci. Source* **8**, 2–5 (2009).
188. Swan, J., Kelsey, A., Crook, B. & Gilbert, E. *Occupational and environmental exposure to bioaerosols from composts and potential health effects- a critical review of published data.* (2003).
189. Brown, A. *et al.* Rapid and responsive monitoring network for bioaerosol emissions: final report. (2009).
190. Dunne, K., Hagen, F., Pomeroy, N., Meis, J. F. & Rogers, T. R. Inter-country Transfer of Triazole-Resistant *Aspergillus fumigatus* on Plant Bulbs. *Clin. Infect. Dis.* **65**, 147–149 (2017).
191. Bromley, M. J. *et al.* Occurrence of azole-resistant species of *Aspergillus* in the UK environment. *J. Glob. Antimicrob. Resist.* **2**, 276–279 (2014).
192. van der Torre, M. H. *et al.* Absence of azole antifungal resistance in *Aspergillus fumigatus* isolated from root vegetables harvested from UK arable and horticultural soils. *J. Fungi* **6**, 1–10 (2020).
193. Henk, D. A. *et al.* Speciation despite globally overlapping distributions in *Penicillium chrysogenum*: The population genetics of Alexander Fleming’s lucky fungus. *Mol. Ecol.* **20**, 4288–4301 (2011).
194. Vieno, M. *et al.* Sensitivities of UK PM2.5 concentrations to emissions reductions. *Atmos. Chem. Phys.* (2015). doi:10.5194/acp-16-265-2016
195. Eiff, M. von *et al.* Pulmonary Aspergillosis: Early Diagnosis Improves Survival. *Respiration* **62**, 341–347 (1995).
196. Finlay, B. J. Global dispersal of free-living microbial eukaryote species. *Science (80-.)*. **296**, 1061–1063 (2002).
197. Sewell, T. R. *et al.* Nonrandom distribution of azole resistance across the global population of *Aspergillus fumigatus*. *MBio* **10**, (2019).
198. Pringle, A. *et al.* Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. *Evolution (N. Y.)*. **59**, 1886–1899 (2005).
199. Bayry, J., Aïmanianda, V., Guijarro, J. I., Sunde, M. & Latgé, J. P. Hydrophobins- unique fungal proteins. *PLoS Pathog.* **8**, 6–9 (2012).
200. Rodney, S. H., Frikkie, C. & Alfred, B. Hyphomycetous fungi spore release induced by air currents and aqueous solution. *African J. Microbiol. Res.* **8**, 2415–2422 (2014).

201. Nanis G. Allam. Protective role of *Aspergillus fumigatus* melanin against ultraviolet (UV) irradiation and *Bjerkandera adusta* melanin as a candidate vaccine against systemic candidiasis. *African J. Biotechnol.* **11**, (2012).
202. Bhabhra, R. & Askew, D. S. Thermotolerance and virulence of *Aspergillus fumigatus*: Role of the fungal nucleolus. *Med. Mycol.* **43**, 87–93 (2005).
203. Vélez-Rodríguez, Z., Torres-Pratts, H. & Maldonado-Ramírez, S. L. Use of Drones to Recover Fungal Spores and Pollen from the Lower Atmosphere. *Caribb. J. Sci.* **50**, 159 (2020).
204. Napoli, C., Marcotrigiano, V. & Montagna, M. T. Air sampling procedures to evaluate microbial contamination: A comparison between active and passive methods in operating theatres. *BMC Public Health* **12**, 1 (2012).
205. Williams, R. H., Ward, E. & McCartney, H. A. Methods for Integrated Air Sampling and DNA Analysis for Detection of Airborne Fungal Spores. *Appl. Environ. Microbiol.* **67**, 2453–2459 (2001).
206. Lemons, A. R., Lindsley, W. G. & Green, B. J. Collection and extraction of occupational air samples for analysis of fungal DNA. *J. Vis. Exp.* **2018**, 1–11 (2018).
207. Clark, C. S., Rylander, R. & Larsson, L. Levels of gram-negative bacteria, *Aspergillus fumigatus*, dust, and endotoxin at compost plants. *Appl. Environ. Microbiol.* **45**, 1501–1505 (1983).
208. Millner, P. D., Bassett, D. A. & Marsh, P. B. Dispersal of *Aspergillus fumigatus* from sewage sludge compost piles subjected to mechanical agitation in open air. *Appl. Environ. Microbiol.* **39**, 10000–11009 (1980).
209. Kothary, M. H., Chase, T. & Macmillan, J. D. Levels of *Aspergillus fumigatus* in air and in compost at a sewage sludge composting site. *Environ. Pollution. Ser. A, Ecol. Biol.* **34**, 1–14 (1984).
210. Grisoli, P. *et al.* Assessment of airborne microorganism contamination in an industrial area characterized by an open composting facility and a wastewater treatment plant. *Environ. Res.* **109**, 135–142 (2009).
211. Recer, G. M., Browne, M. L., Horn, E. G., Hill, K. M. & Boehler, W. F. Ambient air levels of *Aspergillus fumigatus* and thermophilic actinomycetes in a residential neighborhood near a yard-waste composting facility. *Aerobiologia (Bologna)*. **17**, 99–108 (2001).
212. Williams, B., Douglas, P., Roca Barcelo, A., Hansell, A. L. & Hayes, E. Estimating *Aspergillus fumigatus* exposure from outdoor composting activities in England between 2005 and 14. *Waste Manag.* **84**, 235–244 (2019).
213. Kramer, M. N., Kurup, V. P. & Fink, J. N. Allergic Bronchopulmonary Aspergillosis from a Contaminated Dump Site. *Am. Rev. Respir. Dis.* **140**, (1989).
214. Defra. Government Review of Waste Policy in England 2011. *Gov. Waste Policy Rev.* 1–80 (2011).
215. Pearson, C. *et al.* Exposures and health outcomes in relation to bioaerosol emissions from composting facilities: A systematic review of occupational and community studies. *J. Toxicol. Environ. Heal. - Part B Crit. Rev.* **18**, 43–69 (2015).
216. Sykes, P., Jones, K. & Wildsmith, J. D. Managing the potential public health risks from bioaerosol liberation at commercial composting sites in the UK: An analysis of the evidence base. *Resour. Conserv. Recycl.* **52**, 410–424 (2007).

217. Anderson, A. A. New Sampler for the Collection, Sizing and Enumeration of Viable Airborne Particles. *J. Bacteriol.* **76**, 471–484 (1958).
218. Mullins, J., Hutcheson, P. S. & Slavin, R. G. Aspergillus fumigatus spore concentration in outside air: Cardiff and St Louis compared. *Clin. Exp. Allergy* **14**, 351–354 (1984).
219. Opal, S. M. *et al.* Efficacy of Infection Control Measures During a Nosocomial Outbreak of Disseminated Aspergillosis Associated with Hospital Construction. *J. Infect. Dis.* **153**, 634–637 (1986).
220. Dewhurst, A. G., Cooper, M. J., Khan, S. M., Pallett, A. P. & Dathan, J. R. E. Invasive aspergillosis in immunosuppressed patients: Potential hazard of hospital building work. *Br. Med. J.* **301**, 802–804 (1990).
221. Perraud, M. *et al.* Invasive nosocomial pulmonary aspergillosis: Risk factors and hospital building works. *Epidemiol. Infect.* **99**, 407–412 (1987).
222. Goodley, J. M., Clayton, Y. M. & Hay, R. J. Environmental sampling for aspergilli during building construction on a hospital site. *J. Hosp. Infect.* **26**, 27–35 (1994).
223. Brown, H. M. & Jackson, F. A. Aerobiological studies based in Derby. II. Simultaneous pollen and spore sampling at eight sites within a 60 km radius. *Clin. Allergy* **8**, 599–609 (1978).
224. Sadyś, M., Adams-Groom, B., Herbert, R. J. & Kennedy, R. Comparisons of fungal spore distributions using air sampling at Worcester, England (2006–2010). *Aerobiologia (Bologna)*. **32**, 619–634 (2016).
225. Millington, W. M. & Corden, J. M. Long term trends in outdoor Aspergillus/Penicillium spore concentrations in Derby, UK from 1970 to 2003 and a comparative study in 1994 and 1996 with the indoor air of two local houses. *Aerobiologia (Bologna)*. **21**, 105–113 (2005).
226. Jenkins, P. F., Mullins, J., Davies, B. H. & Williams, D. A. The possible role of aero-allergens in the epidemic of asthma deaths. *Clin. Exp. Allergy* **11**, 611–620 (1981).
227. Jarvis, D. & Burney, P. The epidemiology of allergic disease. *BMJ* **316**, 607–610 (1998).
228. Khot, A. & Burn, R. Seasonal variation and time trends of deaths from asthma in England and Wales 1960–82. *Br. Med. J.* **289**, 233–234 (1984).
229. Mackay, T. W., Wathen, C. G., Sudlow, M. F., Elton, R. A. & Caulton, E. Factors Affecting Asthma Mortality in Scotland. *Scott. Med. J.* **37**, (1992).
230. Targonski, P. V., Persky, V. W. & Ramekrishnan, V. Effect of environmental molds on risk of death from asthma during the pollen season. *J. Allergy Clin. Immunol.* **95**, 955–961 (1995).
231. Rhodes, J. *et al.* Tracing patterns of evolution and acquisition of drug resistant Aspergillus fumigatus infection from the environment using population genomics. *bioRxiv* 1–57 (2021).
232. Esri. ArcGIS Pro (Version 2.7.2). (2021). Available at: <https://www.esri.com/en-us/arcgis/products/arcgis-pro/overview>. (Accessed: 19th March 2021)
233. Van't Hek, L. G. *et al.* Successful treatment with voriconazole of invasive aspergillosis in chronic granulomatous disease. *Am. J. Respir. Crit. Care Med.* **157**, 1694–1696 (1998).
234. Yagi, K. *et al.* Invasive pulmonary aspergillosis due to Aspergillus lentulus in an adult patient: A case report and literature review. *J. Infect. Chemother.* **25**, 547–551 (2019).
235. Chowdhary, A., Sharma, C., Hagen, F. & Meis, J. F. Exploring azole antifungal drug resistance in Aspergillus fumigatus with special reference to resistance mechanisms. *Future Microbiol.* **9**,

- 697–711 (2014).
236. Moye-Rowley, W. S. Multiple mechanisms contribute to the development of clinically significant azole resistance in *Aspergillus fumigatus*. *Front. Microbiol.* **6**, 1–6 (2015).
 237. Wei, X. *et al.* Screening and Characterization of a Non-cyp51A Mutation in an *Aspergillus fumigatus* cox10 Strain Conferring Azole Resistance. **61**, 1–14 (2016).
 238. Wang, F. *et al.* Transcription in fungal conidia before dormancy produces phenotypically variable conidia that maximize survival in different environments. *Nat. Microbiol.* (2021). doi:10.1038/s41564-021-00922-y
 239. Douglas, P. *et al.* Use of dispersion modelling for Environmental Impact Assessment of biological air pollution from composting: Progress, problems and prospects. *Waste Manag.* **70**, 22–29 (2017).
 240. Baskaran, A. & Kashef, A. Investigation of air flow around buildings using computational fluid dynamics techniques. *Eng. Struct.* **18**, 861–873 (1996).
 241. Ministry of Housing, C. & L. G. *Land use in England, 2018*. (2020).
 242. Kabir, Z. Tillage or no-tillage: Impact on mycorrhizae. *Can. J. Plant Sci.* **85**, 23–29 (2005).
 243. Chaudhary, V. B., Nolimail, S., Sosa-Hernandez, M. A., Egan, C. & Kastens, J. Trait-based aerial dispersal of arbuscular mycorrhizal fungi. *bioRxiv* 1–9 (2020).
 244. Berger, S., Chazli, Y. El, Babu, A. F. & Coste, A. T. Azole resistance in *Aspergillus fumigatus*: A consequence of antifungal use in agriculture? *Front. Microbiol.* **8**, 1–6 (2017).
 245. Romano, P. V., Krogmann, U., Westendorf, M. L. & Strom, P. F. Small-scale composting of horse manure mixed with wood shavings. *Compost Sci. Util.* **14**, 132–141 (2006).
 246. Viegas, C. *et al.* Fungal contamination of poultry litter: A public health problem. *J. Toxicol. Environ. Heal. - Part A Curr. Issues* **75**, 1341–1350 (2012).
 247. Whitehead, R. *The UK Pesticide Guide 2006*. (2006).
 248. Chawla, S. L., Agnihotri, R., Patel, M. A., Patil, S. & Shah, H. P. Turfgrass: A Billion Dollar Industry. *Natl. Conf. Floric. Rural Urban Prosper. Scenar. Clim. Chang.* 30–35 (2018).
 249. Statista. *Leading destination countries of live trees, plants and flowers exported from the Netherlands in 2019, by export value (in thousand euros)*. (2020).
 250. Environment Agency. *Quality Protocol: Compost - End of waste criteria for the production and use of quality compost from source-segregated biodegradable waste*. (2012).
 251. Renewable Energy Assurance Limited. *Compost/ Biofertiliser Certification Schemes ' Annual Report 2018*. (2018).
 252. HM Government. *Food 2030*. (2010).
 253. Bhogal, A. *et al.* Effect of repeated digestate and compost applications on soil and crop quality. *WRAP DC-Agri Work Packag. 1 Rep.* 1–47 (2016).
 254. Déportes, I., Benoit-Guyod, J. L. & Zmirou, D. Hazard to man and the environment posed by the use of urban waste compost: a review. *Sci. Total Environ.* **172**, 197–222 (1995).
 255. Sabino, R. *et al.* The role of occupational *Aspergillus* exposure in the development of diseases. *Med. Mycol.* **57**, S196–S205 (2019).

256. Eduard, W. Fungal spores: A critical review of the toxicological and epidemiological evidence as a basis for occupational exposure limit setting. *Crit. Rev. Toxicol.* **39**, 799–864 (2009).
257. Viegas, C. *et al.* Aspergillus spp. prevalence in different Portuguese occupational environments: What is the real scenario in high load settings? *J. Occup. Environ. Hyg.* **14**, 771–785 (2017).
258. Schantora, A. L. *et al.* Prevalence of Work-Related Rhino-Conjunctivitis and Respiratory Symptoms Among Domestic Waste Collectors. *Environment Exposure to Pollutants* (2014).
259. Velasco Garrido, M., Bittner, C., Harth, V. & Preisser, A. M. Health status and health-related quality of life of municipal waste collection workers - A cross-sectional survey. *J. Occup. Med. Toxicol.* **10**, 1–7 (2015).
260. Hoffmeyer, F. *et al.* Prevalence of and relationship between rhinoconjunctivitis and lower airway diseases in compost workers with current or former exposure to organic dust. *Ann. Agric. Environ. Med.* **21**, 705–711 (2014).
261. Van Kampen, V. *et al.* Symptoms, spirometry, and serum antibody concentrations among compost workers exposed to organic dust. *J. Toxicol. Environ. Heal. - Part A Curr. Issues* **75**, 492–500 (2012).
262. Hambach, R. *et al.* Work-related health symptoms among compost facility workers: a cross-sectional study. *Arch. Public Heal.* **70**, 2–6 (2012).
263. Athanasiou, M., Makrynos, G. & Dounias, G. Respiratory health of municipal solid waste workers. *Occup. Med. (Chic. Ill)*. **60**, 618–623 (2010).
264. Allmers, H., Huber, H. & Baur, X. Two year follow-up of a garbage collector with allergic bronchopulmonary aspergillosis (ABPA). *Am. J. Ind. Med.* **37**, 438–442 (2000).
265. Poole, C. J. M. & Wong, M. Allergic bronchopulmonary aspergillosis in garden waste (compost) collectors-occupational implications. *Occup. Med. (Chic. Ill)*. **63**, 517–519 (2013).
266. Bünger, J., Schappler-Scheele, B., Hilgers, R. & Hallier, E. A 5-year follow-up study on respiratory disorders and lung function in workers exposed to organic dust from composting plants. *Int. Arch. Occup. Environ. Health* **80**, 306–312 (2007).
267. Vincken, W. & Roels, P. Hypersensitivity pneumonitis due to Aspergillus fumigatus in compost. *Thorax* **39**, 74–75 (1984).
268. Cushen, B. *et al.* High prevalence of obstructive lung disease in non-smoking farmers: The Irish farmers lung health study. *Respir. Med.* **115**, 13–19 (2016).
269. Eduard, W., Pearce, N. & Douwes, J. Chronic bronchitis, COPD, and lung function in farmers: The role of biological agents. *Chest* **136**, 716–725 (2009).
270. Vergnenegre, A. *et al.* Work related distal airway obstruction in an agricultural population. *Occup. Environ. Med.* **52**, 581–586 (1995).
271. Dalphin, J. C. *et al.* Prevalence and risk factors for chronic bronchitis and farmer's lung in French dairy farmers. *Br. J. Ind. Med.* **50**, 941–944 (1993).
272. Schønheyder, H., Møller-Hansen, C., Andersen, P. & Stenderup, A. Serum antibodies to Aspergillus fumigatus in Danish farmers. *Med. Mycol.* **23**, 93–99 (1985).
273. Louhelainen, K. J. Farmers' exposure to dusts and gases in dairy farms. (1997).
274. Sabino, R., Faísca, V. M., Carolino, E., Veríssimo, C. & Viegas, C. Occupational exposure to

- Aspergillus by swine and poultry farm workers in Portugal. *J. Toxicol. Environ. Heal. - Part A Curr. Issues* **75**, 1381–1391 (2012).
275. Patterson, R., Sommers, H. & Fink, J. N. Farmer's lung following inhalation of *Aspergillus flavus* growing in mouldy corn. *Clin. Exp. Allergy* **4**, 79–86 (1974).
 276. Sakamoto, T. *et al.* An onion farmer with a case of hypersensitivity pneumonitis caused by *Aspergillus niger*. *Respir. Med. Case Reports* **23**, 60–62 (2018).
 277. Flood-Page, P. A case of allergic bronchopulmonary aspergillosis in a 43-year-old farmer following single high level exposure to organic dust with symptomatic remission and radiological resolution after early diagnosis and treatment. *Oxford Med. Case Reports* **2018**, 42–44 (2018).
 278. Dupreza, M., Soumagnea, T., Maitreb, J., Reboux, G. & Dalphin, J.-C. A case of farmer's lung associated with allergic bronchopulmonary aspergillosis. *Rev. Mal. Respir.* **37**, 80–85 (2020).
 279. Baur, X., Hahn, D. & Weiss, W. Allergic bronchopulmonary aspergillosis with hay dust-induced alveolitis and bronchial asthma. *Dtsch. Medizinisch Wochenschrift* **113**, 1105–8 (1988).
 280. Oxford Economics. *The Economic Impact of Ornamental Horticulture and Landscaping in the UK - A Report for the Ornamental Horticulture Round Table Group.* (2018).
 281. Defra. *Household Waste Prevention Evidence Review- A report for Defra's Waste and Resources Evidence Programme.* (2009).
 282. Chowdhary, A., Kathuria, S., Xu, J. & Meis, J. F. Emergence of Azole-Resistant *Aspergillus fumigatus* Strains due to Agricultural Azole Use Creates an Increasing Threat to Human Health. *PLoS Pathog.* **9**, 3–7 (2013).
 283. Brown, J. E., Masood, D., Couser, J. I. & Patterson, R. Hypersensitivity pneumonitis from residential composting: residential composter's lung. *Ann. allergy, asthma Immunol.* **74**, 45–7 (1995).
 284. Office for National Statistics. *UK Natural Capital: Urban Accounts. Statistical Bulletin* (2019).
 285. Statista. *Share of adult population gardening in free time in England in 2017/18, by age.* (2020).
 286. Cavling Arendrup, M., Ronan O'Driscoll, B., Petersen, E. & Denning, D. W. Acute pulmonary aspergillosis in immunocompetent subjects after exposure to bark chippings. *Scand. J. Infect. Dis.* **38**, 945–949 (2006).
 287. Batard, E., Renaudin, K., Morin, O., Desjars, P. & Germaud, P. Fatal acute granulomatous pulmonary aspergillosis in a healthy subject after inhalation of vegetal dust. *Eur. J. Clin. Microbiol. Infect. Dis.* **22**, 357–359 (2003).
 288. Jung, N. *et al.* Gardening can induce pulmonary failure: *Aspergillus* ARDS in an immunocompetent patient, a case report. *BMC Infect. Dis.* **14**, 1–3 (2014).
 289. Russell, K., Broadbridge, C., Murray, S., Waghorn, D. & Mahoney, A. Gardening can seriously damage your health. *Lancet* **371**, 2056 (2008).
 290. Zuk, L. A., King, D., Zakhour, H. D. & Delaney, J. C. Locally invasive pulmonary aspergillosis occurring in a gardener: an occupational hazard? *Thorax* **44**, 678–679 (1989).
 291. Jones, J. L., Krueger, A., Schulkin, J. & Schantz, P. M. Toxoplasmosis prevention and testing in pregnancy, survey of obstetrician-gynaecologists. *Zoonoses Public Health* **57**, 27–33 (2010).

292. Zhang, J. *et al.* Dynamics of *Aspergillus fumigatus* in Azole Fungicide-Containing Plant Waste in the Netherlands (2016-2017). *Appl. Environ. Microbiol.* **87**, 1–12 (2021).
293. Santoro, K. *et al.* Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* isolates from organic substrates with special emphasis on compost. *Pest Manag. Sci.* **73**, 2481–2494 (2017).
294. Pugliese, M., Matić, S., Prethi, S., Gisi, U. & Gullino, M. L. Molecular characterization and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* from orange-based compost. *PLoS One* **13**, 1–18 (2018).
295. Ahangarkani, F. *et al.* First azole-resistant *Aspergillus fumigatus* isolates with the environmental TR46/Y121F/T289A mutation in Iran. *Mycoses* **63**, 430–436 (2020).
296. Hagiwara, D. Isolation of azole-resistant *Aspergillus fumigatus* from imported plant bulbs in Japan and the effect of fungicide treatment. *J. Pestic. Sci.* **45**, 147–150 (2020).
297. Cho, S. Y. *et al.* Epidemiology and antifungal susceptibility profile of *Aspergillus* species: Comparison between environmental and clinical isolates from patients with hematologic malignancies. *J. Clin. Microbiol.* **57**, 1–13 (2019).
298. Alvarez-Moreno, C. *et al.* Azole-resistant *Aspergillus fumigatus* harboring TR34/L98H, TR46/Y121F/T289A and TR53 mutations related to flower fields in Colombia. *Sci. Rep.* **7**, (2017).
299. Majima, H., Teppei, A., Watanabe, A. & Kamei, K. D430G mutation of *cyp51A* in *Aspergillus fumigatus* causes azole-resistance. in *9th Advances Against Aspergillosis and Mucormycosis* 90 (2020).
300. Howard, S. J. *et al.* Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* **15**, 1068–1076 (2009).
301. Semenza, J. C. & Menne, B. Climate change and infectious diseases in Europe. *Lancet Infect. Dis.* **9**, 365–375 (2009).
302. Epstein, P. R. Climate change and infectious disease: Stormy weather ahead? *Epidemiology* **13**, 373–375 (2002).
303. Lafferty, K. D. The ecology of climate change and infectious diseases. *Ecology* **90**, 888–900 (2009).
304. Lindgren, E., Andersson, Y., Suk, J. E., Sudre, B. & Semenza, J. C. Public health: Monitoring EU emerging infectious disease risk due to climate change. *Science (80-.)*. **336**, 418–419 (2012).
305. Patz, J. A. *et al.* Climate change and infectious diseases. in *Climate Change and Human Health* 103–132 (2003). doi:10.1186/s40985-016-0035-2
306. Wu, X., Lu, Y., Zhou, S., Chen, L. & Xu, B. Impact of climate change on human infectious diseases: Empirical evidence and human adaptation. *Environ. Int.* **86**, 14–23 (2016).
307. Yun, J. *et al.* Association between the ambient temperature and the occurrence of human *Salmonella* and *Campylobacter* infections. *Sci. Rep.* **6**, 1–7 (2016).
308. Islam, M. S. *et al.* Effects of local climate variability on transmission dynamics of cholera in Matlab, Bangladesh. *Trans. R. Soc. Trop. Med. Hyg.* **103**, 1165–1170 (2009).
309. MacFadden, D. R., McGough, S. F., Fisman, D., Santillana, M. & Brownstein, J. S. Antibiotic resistance increases with local temperature. *Nat. Clim. Chang.* **8**, 510–514 (2018).

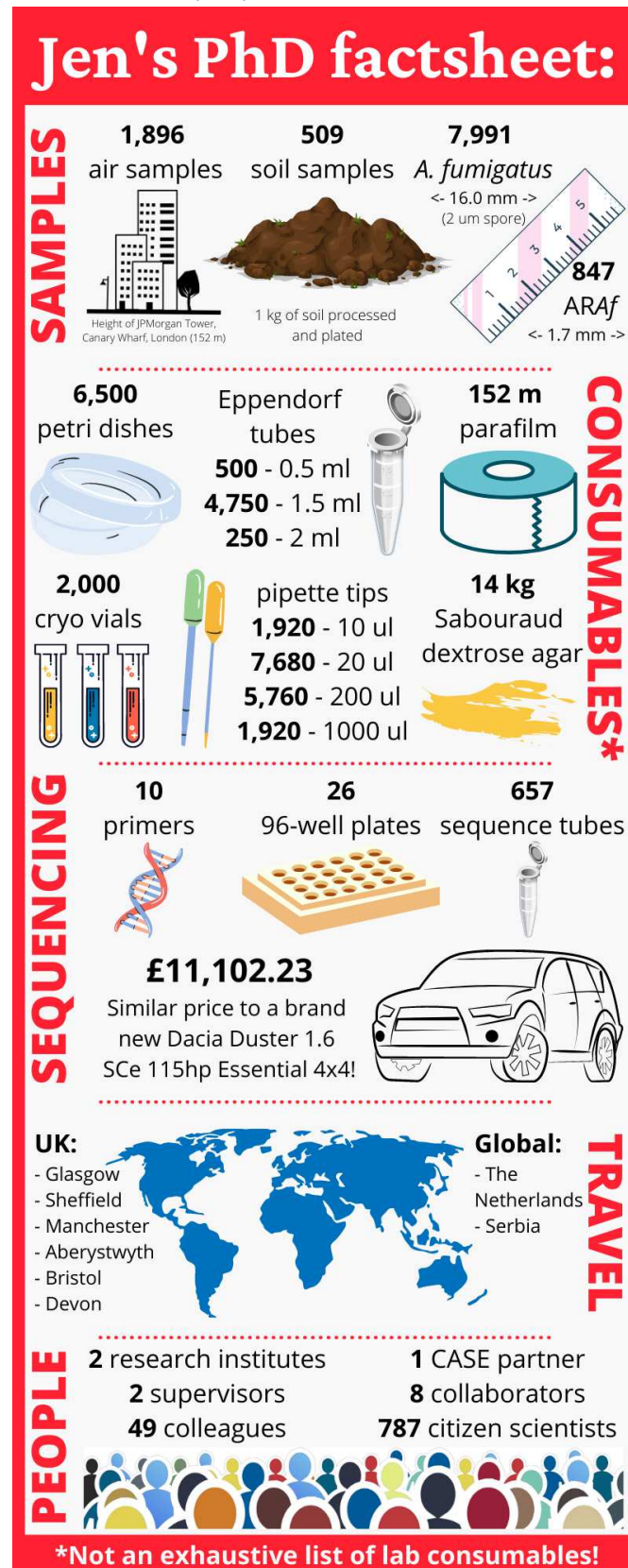
310. Bebbber, D. P., Ramotowski, M. A. T. & Gurr, S. J. Crop pests and pathogens move polewards in a warming world. *Nat. Clim. Chang.* **3**, 985–988 (2013).
311. Griffin, D. W. Atmospheric movement of microorganisms in clouds of desert dust and implications for human health. *Clin. Microbiol. Rev.* **20**, 459–477 (2007).
312. Williams, P. L., Sable, D. L., Mendez, P. & Smyth, L. T. Symptomatic coccidioidomycosis following a severe natural dust storm. An outbreak at the Naval Air Station, Lemoore, Calif. *Chest* **76**, 566–570 (1979).
313. Schneider, E. *et al.* A Coccidioidomycosis Outbreak Following the Northridge, Calif, Earthquake. *JAMA* **277**, 904–908 (1997).
314. Torres Castillo, N. E. *et al.* Impact of climate change and early development of coffee rust – An overview of control strategies to preserve organic cultivars in Mexico. *Sci. Total Environ.* **738**, (2020).
315. Gateau-Rey, L., Tanner, E. V. J., Rapidel, B., Marelli, J. P. & Royaert, S. Climate change could threaten cocoa production: Effects of 2015–16 El Niño-related drought on cocoa agroforests in Bahia, Brazil. *PLoS One* **13**, 1–17 (2018).
316. Ortega Andrade, S., Pérez, G. T., Fera, T. P. & Muñoz, J. Climate change and the risk of spread of the fungus from the high mortality of Theobroma cocoa in Latin America. *Neotrop. Biodivers.* **3**, 30–40 (2017).
317. Harvell, C. D. *et al.* Climate warming and disease risks for terrestrial and marine biota. *Science* (80-.). **296**, 2158–2162 (2002).
318. Sutherland, M. L., Pearson, S. & Brasier, C. M. The influence of temperature and light on defoliation levels of elm by Dutch elm disease. *Phytopathology* **87**, 576–581 (1997).
319. Houston, D. R. & Valentine, H. T. Beech bark disease: the temporal pattern of cankering in aftermath forests of Maine. *Can. J. For. Res.* (1988).
320. Luck, J. *et al.* Climate change and diseases of food crops. *Plant Pathol.* **60**, 113–121 (2011).
321. Milus, E. A., Seyran, E. & Mcnew, R. Aggressiveness of Puccinia striiformis f. sp. tritici isolates in the South-Central United States. *Plant Dis.* 847–852 (2006).
322. Luo, Y., Teng, P. S., Fabellar, N. G. & Tebeest, D. O. The effects of global temperature change on rice leaf blast epidemics: A simulation study in three agroecological zones. *Agric. Ecosyst. Environ.* **68**, 187–196 (1998).
323. Bebbber, D. P., Holmes, T. & Gurr, S. J. The global spread of crop pests and pathogens. *Glob. Ecol. Biogeogr.* **23**, 1398–1407 (2014).
324. Casadevall, A. Climate change brings the specter of new infectious diseases. *J. Clin. Invest.* **130**, 553–555 (2020).
325. Greer, A., Ng, V. & Fisman, D. Climate change and infectious diseases in North America: The road ahead. *CMAJ* **178**, 715–722 (2008).
326. Barrozo, L. V. *et al.* Climate and acute/subacute paracoccidioidomycosis in a hyper-endemic area in Brazil. *Int. J. Epidemiol.* **38**, 1642–1649 (2009).
327. Robert, V. A. & Casadevall, A. Vertebrate endothermy restricts most fungi as potential pathogens. *J. Infect. Dis.* **200**, 1623–1626 (2009).
328. McLean, M. A., Angilletta, M. J. & Williams, K. S. If you can't stand the heat, stay out of the

- city: Thermal reaction norms of chitinolytic fungi in an urban heat island. *J. Therm. Biol.* **30**, 384–391 (2005).
329. M.Tibbett, F.E.Sanders, J.W.G.Cairney & J.R.Leake. Temperature regulation of extracellular proteases in ectomycorrhizal fungi (*Hebeloma* spp.) grown in axenic culture. *Mycol. Res.* **103**, 707–714 (1999).
 330. Leach, M. D., Tyc, K. M., Brown, A. J. P. & Klipp, E. Modelling the regulation of thermal adaptation in *Candida albicans*, a major fungal pathogen of humans. *PLoS One* **7**, 1–14 (2012).
 331. Joffrion, T. M. & Cushion, M. T. Sterol biosynthesis and sterol uptake in the fungal pathogen *Pneumocystis carinii*. *FEMS Microbiol. Lett.* **311**, 1–9 (2010).
 332. Dupont, S. *et al.* Ergosterol biosynthesis: A fungal pathway for life on land? *Evolution (N. Y.)*. **66**, 2961–2968 (2012).
 333. Trösken, E. R. *et al.* Comparison of lanosterol-14 α -demethylase (CYP51) of human and *Candida albicans* for inhibition by different antifungal azoles. *Toxicology* **228**, 24–32 (2006).
 334. Buttke, T. M., Jones, S. D. & Bloch, K. Effect of sterol side chains on growth and membrane fatty acid composition of *Saccharomyces cerevisiae*. *J. Bacteriol.* **144**, 124–130 (1980).
 335. Zhou, D. *et al.* Extensive Genetic Diversity and Widespread Azole Resistance in Greenhouse Populations of *Aspergillus fumigatus* in Yunnan, China . *mSphere* **6**, (2021).
 336. Robertson, J. C. & Hazel, J. R. Cholesterol content of trout plasma membranes varies with acclimation temperature. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **269**, (1995).
 337. Cuculescu, M., Hyde, D. & Bowler, K. Temperature acclimation of marine crabs: Changes in plasma membrane fluidity and lipid composition. *J. Therm. Biol.* **20**, 207–222 (1995).
 338. Hassett, R. P. & Crockett, E. L. Habitat temperature is an important determinant of cholesterol contents in copepods. *J. Exp. Biol.* **212**, 71–77 (2009).
 339. Reynolds, A. M., Lee, R. E. & Costanzo, J. P. Membrane adaptation in phospholipids and cholesterol in the widely distributed, freeze-tolerant wood frog, *Rana sylvatica*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **184**, 371–383 (2014).
 340. Weinstein, R. N., Montiel, P. O. & Johnstone, K. Influence of growth temperature on lipid and soluble carbohydrate synthesis by fungi isolated from fellfield soil in the maritime Antarctic. *Mycologia* **92**, 222–229 (2000).
 341. Hu, Z. *et al.* Recent Advances in Ergosterol Biosynthesis and Regulation Mechanisms in *Saccharomyces cerevisiae*. *Indian J. Microbiol.* **57**, 270–277 (2017).
 342. Kamthan, A., Kamthan, M. & Datta, A. Expression of C-5 sterol desaturase from an edible mushroom in fission yeast enhances its ethanol and thermotolerance. *PLoS One* **12**, 1–20 (2017).
 343. Kodedová, M. & Sychrová, H. Changes in the sterol composition of the plasma membrane affect membrane potential, salt tolerance and the activity of multidrug resistance pumps in *Saccharomyces cerevisiae*. *PLoS One* **10**, 1–19 (2015).
 344. Office for National Statistics. Overview of the UK population: January 2021. (2021). Available at: <https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/articles/overviewoftheukpopulation/january2021>. (Accessed: 20th July 2021)

345. Johnson, R. J., Bradbury, L. L., Martin, K. & Neuberger, J. Organ donation and transplantation in the UK-The last decade: A report from the UK national transplant registry. *Transplantation* **97**, 1–27 (2014).
346. Maddams, J., Utley, M. & Møller, H. Projections of cancer prevalence in the United Kingdom, 2010-2040. *Br. J. Cancer* **107**, 1195–1202 (2012).
347. Alanio, A., Dellièrè, S., Fodil, S., Bretagne, S. & Mégarbane, B. Prevalence of putative invasive pulmonary aspergillosis in critically ill patients with COVID-19. *Lancet Respir. Med.* **8**, e48–e49 (2020).
348. Göttlich, E. *et al.* Fungal flora in groundwater-derived public drinking water. *Int. J. Hyg. Environ. Health* **205**, 269–279 (2002).
349. Parveen, S., Lanjewar, S., Sharma, K. & Kutti, U. Isolation of fungi from the surface water of river. *J. Exp. Sci.* **2**, 58–59 (2011).
350. Haas, D. *et al.* Comparative investigation of airborne culturable microorganisms in sewage treatment plants. *Cent. Eur. J. Public Health* **10**, 6–10 (2002).
351. Raut, A. & Huy, N. T. Rising incidence of mucormycosis in patients with COVID-19: another challenge for India amidst the second wave? *Lancet Respir. Med.* **2600**, 2600 (2021).
352. Schauwvlieghe, A. F. A. D. *et al.* High-dose posaconazole for azole-resistant aspergillosis and other difficult-to-treat mould infections. *Mycoses* **63**, 122–130 (2020).
353. Panackal, A. A., Bennett, J. E. & Williamson, P. R. Treatment options in Invasive Aspergillosis. *Curr. Treat. Options Infect. Dis.* **23**, 1–7 (2014).
354. Steinberg, G. & Gurr, S. J. Fungi, fungicide discovery and global food security. *Fungal Genet. Biol.* **144**, 103476 (2020).
355. Defra. *Agriculture in the United Kingdom 2019*. (2020).
356. Singer, A. C., Kirchhelle, C. & Roberts, A. P. (Inter)nationalising the antibiotic research and development pipeline. *Lancet Infect. Dis.* **20**, 54–62 (2020).

Appendices

Appendix 1: Infographic of thesis including samples collected, consumables used, sequencing undertaken, places travelled to and people involved.



Appendix 2: Importance of fungi in nature, food, medicine, industry and bioremediation.

Fungi in nature

Plant growth

Mycorrhizal fungi form symbiotic relationships with plants by colonising their root systems and providing them with nutrients, such as nitrogen and phosphate, and improving their water uptake capacity in return for a share of the carbon they photosynthesise [1]. Around 80% of plant species form these mycorrhizal interactions [2], which can be with arbuscular mycorrhizals (AM) that penetrate intracellularly into the root cortex or with ectomycorrhizals (ECM) that remain intercellular [1]. Of the estimated 450 Gt C stored in plant biomass, it is estimated that ~241 Gt C is in plants associated with AM and ~100 Gt C in those associated with ECM, compared to 29 Gt C in non-mycorrhizal plants, due to the benefits of these fungi for plant growth [3]. AM are more common, forming ~65% of associations, and also receive more attention because their host plants include many important crops such as rice, corn, soybean and wheat [1]. In an agricultural setting, AM enhance plant growth, increase their resistance to drought and disease, decrease need for phosphorus and nitrogen fertilisers [4] and alleviate stresses to the plant such as pollution and heavy metal contamination [5]. Incredibly, mycorrhizal networks (MNs) can form between plants allowing them to transfer nutrients and warn each other of damage by herbivores or pathogens, resulting in up-regulation of stress responses in undamaged neighbours [6]. ECM associate with <5% of plant species and are found predominantly in forest biomes where research suggests their MNs allow tree seedlings to recognise kin and preferentially transfer nutrients to siblings over non-siblings [7].

Nutrient recycling

Saprotrophic fungi play an important role in the ecosystem decomposing organic matter and recycling carbon and nutrients into the soil, such that it is likely that life on earth would cease after a few decades without these fungi because carbon and nutrients would become unavailable to the systems requiring them [8]. Through their decomposing activities fungi produce an organic material called humus, which increases the structure and water-holding capacity of soil [8] and acts as the main carbon reservoir in the biosphere, holding an estimated 1600×10^{15} Gt C [9].

Early life on Earth

It is likely that the mycorrhizal and saprophytic qualities of fungi aided the first plants in colonising land. The fossil record shows that the earliest land-colonising plants lacked roots and formed symbioses with fungi more than 475 mya, and that together the plants and fungi reduced the elevated carbon dioxide (CO₂) levels in the atmosphere by silicate weathering and increased availability of phosphorus through biotic weathering of rocks [10]. These early landscapes were dominated by giant fungi, called prototaxites, that could grow to up to 8.8 m tall and 1 m in diameter but it is not yet known whether these were saprophytes or symbionts [11].

Pest control

At present, 40% of crops are lost to pests including insects, pathogens and weeds [12]. It is estimated that insects alone consume 5-20% of major crops and this is set to increase with rising global temperatures due to climate change [13]. Pest control has relied heavily on chemical interventions since the late 1940s, with insecticides accounting for 44% of global usage, herbicides for 30% and fungicides for 21% [14], yet there is great interest in using fungi as biopesticides. The first record of

using fungi to control a pest occurred in Russia in 1888 when *Metarhizium anisopliae* was mass produced and sprayed on beet fields to control beet weevil [15]. Since the 1960s, 171 products have been licensed using at least 12 species of entomopathogenic fungi as the active ingredient to control 48 families of insects, with *Beauveria bassiana* and *M. anisopliae* found in 64% of products [15]. Mycoherbicides can be applied to crops to reduce levels of weeds that compete with or parasitise crops and some successful examples include a 95% reduction in *Acacia saligna* density in South Africa by *Uromycladium tepperianum* from 1987-1989 and the rehabilitation of 50,000 hectares of pasture in Hawaii when *Entyloma ageratinae* reduced the population of *Ageratina riparia* to less than 5% within 9 months [16]. When *Puccinia chondrillina* was released to control the weed *Chondrilla juncea* it was estimated that the benefits were at least 100 times greater than the costs involved [17]. Fungi can even be effective against other fungi: 90% of mycofungicides contain *Trichoderma* sp. to control *Botrytis cineria*, *Fusarium*, *Pythium* and *Rhizoctonia* and the remainder employ *Ampelomyces quisqualis* against powdery mildew, *Chaetomium globosum* against root rot disease and *Glilotadium virens* against soilborne pathogens [18].

Fungi as food and in the food industry

Edible mushrooms

Edible mushrooms are a nutritional food source as they are naturally high in vitamin D, generating enough vitamin D to provide an adult's recommended daily requirement in a single 100g serving [19]. They are also low in calories, carbohydrates, calcium and sodium, and contain large numbers of vitamins and minerals [20]. When people talk about "mushrooms" they are referring to macrofungi, which are the fruiting bodies we can see, usually above ground or attached to trees, that fungi produce to disperse spores and are connected to a network of mycelia below. (As opposed to microfungi that are invisible to the naked eye until their hyphae or spores number in their millions.) Commercial mushroom production is on the rise and has increased greater than 25-fold in the past 35 years, with *Agaricus bisporus* (button mushroom), *Pleurotus* (oyster mushroom) and *Lentinula edodes* (shiitake mushroom) accounting for 85% of worldwide consumption [21]. Truffles, belonging to the genus *Tuber*, are the most expensive edible mushroom and fetch prices in the region of €600-6000 per kilogram [22]. As mushroom cultivation is low-cost and has low environmental impact it is being explored as a solution to global poverty, malnutrition and environmental degradation in developing countries [23].

Mycoprotein

Mycoprotein is a food ingredient produced by *Fusarium venenatum* that is high in protein and fibre, and low in fat, cholesterol, sodium, and sugar [24]. It is sold as Quorn™ in 19 countries worldwide and marketed as a meat-alternative, with a texture similar to chicken and requiring 10 times less carbon, water and land use than beef [24]. In 2019, Quorn Foods reported its annual global sales as £220 million with demand set to rise as more people switch to non-meat diets.

Yeast as fermenters

The use of yeast, specifically *Saccharomyces cerevisiae*, in fermentation reactions to produce food and beverages is the oldest and most economically important biotechnology [25]. Baking and brewing using yeast were first depicted in Egyptian hieroglyphs from 4,000 years ago [26] and today it is used in winemaking, beer brewing and bread baking. In the UK, the alcohol industry alone contributes approximately 2.5% of total gross domestic product (GDP) to national income [27] and it is estimated that in the US 3% of the workforce is employed in yeast-based production [28]. The importance of

yeast comes from its ability to convert sugars into carbon dioxide and ethanol under both aerobic and anaerobic conditions, which is what causes bread dough to rise and makes wine and beer alcoholic [29]. Baker's yeast and brewer's yeast are different strains of *S. cerevisiae*.

Moulds as fermenters

Many components of Asian cuisine are produced by fermentation using moulds. Examples include fermentation of wheat and soybean by *Aspergillus oryzae* or *Aspergillus sojae* into soy sauce [30] and fermentation of rice by *A. oryzae* and *S. cerevisiae* into sake [25]. In the West, moulds are used in cheese production; either using *Penicillium camemberti* to produce a rind on soft cheeses like Camembert and Brie or using *Penicillium roqueforti* to produce blue-veined cheeses such as Roquefort, Stilton, and Gorgonzola [25]. *Penicillium* moulds also colonise the surface of dry-cured meats, enhancing their flavour and aroma and increasing their shelf-life by preventing spoilage by other microbes [31].

Fungi in medicine

Antimicrobials

The most famous example of fungi in medicine is the discovery of penicillin by Sir Alexander Fleming in 1928. Fleming was working on staphylococcal bacteria when some of his plates became contaminated with mould spores and he noticed an inhibition zone around these colonies in which the bacteria no longer grew. He nicknamed his finding 'mould juice' and later named it penicillin when he identified the mould as belonging to the genus *Penicillium*. Fleming found penicillin to be active against all Gram-positive bacteria and published his findings in 1929 [32] but little notice was taken until 1940 when two scientists, Howard Florey and Ernst Chain, began mass-producing penicillin for use during World War II (WWII) [33]. Fleming, Florey and Chain were awarded the Nobel Prize for Physiology or Medicine in 1945 and the discovery of penicillin triggered the beginning of the antibiotic era, which revolutionised the treatment of infectious diseases worldwide and began transitioning the leading causes of death in developed countries away from communicable diseases.

In the 1950s another natural antibiotic, Cephalosporin C, was discovered in an *Acremonium* species collected off the Sardinian coast [34] and represented the first of many fungal antibiotic compounds isolated from a marine environment [35]. Other fungal metabolites discovered to have antibacterial properties include itaconic acid from *Aspergillus itaconicus* (1931) [36], fumagillin from *Aspergillus fumigatus* (1949) [37], fusidic acid from *Fusarium sp.* (1962) [38], alamethicin from *Trichoderma viride* (1966) [39], aphidicolin from *Cephalosporium aphidicola* (1973) [40], Mycotoxin MT81 from *Penicillium nigricans* (1992) [41] and nigrosporins A and B from *Nigrospora oryzae* (1997) [42]. In the arms race against their microbial peers, fungi have also produced compounds effective against other fungi (griseofulvin from *Penicillium griseofulvum* [43]; *strobilurins* from *Strobilurus tenacellus* [44]; *echinocandins* from *Aspergillus nidulans* [45]; *papulacandins* from *Papularia sphaerosperma* [46]), viruses (brefeldin A and verrucarins A [47]) and protozoa (hypocrellins A and B [48]; puberulic acid [49]). The search for novel antimicrobials is ongoing and it is likely that we have just scratched the surface of what fungi can offer us from their millions of years of coevolution with these microbes in the environment.

Lowering cholesterol

Statins, or HMG-CoA reductase inhibitors, lower levels of low-density lipoproteins (LDL) cholesterol in the blood and significantly reduce the risk of death from myocardial infarction and coronary heart disease (CHD) [50]–[52]. Mevastatin and compactin were the first statins discovered; mevastatin was

isolated from *Penicillium citrinum* in 1976 [53] and compactin from *Penicillium brevicompactum* in 1978 [54] but lovastatin was the first statin to be approved by the Food and Drug Administration (FDA) and made available for pharmaceutical use [55]. Lovastatin is now produced commercially using *Aspergillus terreus* and is one of the highest selling drugs in the United States (US) with sales totalling \$11.6 billion by 2011 [56].

Antidepressants

A genus of mushrooms called psilocybes produce psilocybin, and are known as “magic mushrooms” because of their psychedelic effects. Psilocybin binds non-selectively to serotine receptors and has been found to have long-term antidepressant effects in people with treatment-resistant depression [57] as well as alleviating anxiety in end-of-life patients [58], treating tobacco [59] and alcohol [60] addiction and reducing symptoms of obsessive-compulsive disorder (OCD) [61].

Anticancer

The statins lovastatin and compactin, and the synthetic derivative simvastatin, also have inhibitive effects against cancers including acute myeloid leukaemia (AML), lung, ovarian, breast, liver, cervical cancers, melanomas and more [62]. Many macrofungi that have been used in traditional medicines since as far back as 3000BC produce active biocompounds that have immunomodulatory effects, and have anticancer effects by up- or downregulating the body’s innate and adaptive immune responses [63]. Some examples are the polysaccharide lentinan produced by *Lentinula edodes* (Shiitake mushroom) [64], ganoderic acids produced by *Ganoderma lucidum* (Reishi mushroom) [65], ergosterols produced by *Cordyceps sinensis* (a fungus that infects sphinx moth larvae in the Himalayas) [66] and polysaccharide peptide (PSP) produced by *Coriolus versicolor* (Turkey tail) [67]. Despite many promising compounds only a few have made it to clinical trials, likely due to challenges in scaling up production [68], and, as yet, none have been approved for clinical use but research is ongoing.

Immunosuppressants

Cyclosporine, produced by *Tolypocladium inflatum*, was being investigated in 1970 as a potential antifungal when it was also tested for cytostatic, antiviral and immunosuppressive activity and was found to suppress cell- and antibody-mediated immunity and chronic inflammation [69]. It became the first metabolite produced by a microbe to be licensed for use as an immunosuppressant [69] and is now used extensively in organ transplant patients to reduce graft rejection. Other immunosuppressants produced by fungi are mizoribine from *Penicillium brefeldianum* [70], mycophenolic acid from *Penicillium brevicompactum* [71] and subglutinols from *Fusarium subglutinans* [72] and are used to treat autoimmune diseases such as Crohn’s disease, rheumatoid arthritis, lupus and psoriasis, as well as for organ transplants.

Fungi in industry

Clothing

During WWII, the uniforms and tents of Americans serving in the Pacific were disintegrating, which was found to be due to the fungus *Trichoderma reesei* producing cellulases that were breaking down cellulose fibres in the fabrics [73]. Now cellulases, commercially produced by *T. reesei* and *Aspergillus oryzae*, account for approximately 14% of the global enzyme market [74] and are used to give denim fabric a stone-washed look [75] and eliminate hydrogen peroxide after it’s been used to bleach cotton [76]. Lipases, commercially produced by *Aspergillus niger*, account for nearly 10% of the global enzyme market [77] and are used in tanneries to hydrolyse fats, oils and greases present in hides and skins

[78]. Catalases and lipases produced by the thermophilic fungus *Humicola insolens* are added to biological washing powders to breakdown fat stains [79] and to remove cotton threads from the surface of garments to make them feel smoother.

Pigments produced by fungi offer an environmentally-friendly alternative to artificial dyes used in the textiles industry. Wood staining fungi are perfect candidates as the pigments they produce naturally interact with cellulose fibres and pigments from *Penicillium murcianum*, *Talaromyces australis*, *Monascus purpureus* and *Dermocybe sanguinea*, amongst others, have been successfully used to dye fabrics [80]–[82].

Paper manufacture

The paper industry produced 188 million tons of pulp in 2018 [83]; a process that involves wood pulping followed by pulp bleaching. Pulping is the breakdown of raw materials containing lignocellulose, usually wood, into cellulose fibres and is traditionally done mechanically or chemically, using sodium hydroxide or sodium sulphite [84]. Biopulping, the name given to the breakdown of lignocellulose by extracellular enzymes produced by white, brown and soft rot fungi, would reduce the energy consumption and chemical input of traditional pulping methods [85] but has played a relatively minor role due to difficulties in scaling-up enzyme production. The biggest application of fungal enzymes in the paper industry is xylanases, produced by *Trichoderma reesei*, *Thermomyces lanuginosus* and *Aureobasidium pullulans*, that are used in pre-bleaching of pulp as an environmentally-friendly alternative to chlorine [86].

Plastics manufacture

With growing concerns about sustainability, environmental damage and energy usage, chemical companies are looking to move away from chemicals produced from fossil fuels towards those produced from renewable biomass [87]. One such chemical is itaconic acid, produced commercially from *Aspergillus terreus* grown on sugar, that has a global yield of 80,000 tons and can be used to make plastics, rubbers, synthetic fibres and resins, surfactants and oil additives [88]. In its commitment to circular economy, LEGO™ has now started using itaconic acid to make its tree, leaves and bush elements [89].

Biodiesel

Biodiesel is a type of biofuel, produced when lipids in biomass are converted into fatty acid methyl esters (FAMES) that have the equivalent energy content of petroleum diesel and offer a sustainable alternative [90]. This conversion is done by oleaginous organisms that can accumulate lipids intracellularly, including several fungal species: *Cunninghamella echinulata* [91], *Mortierella alpina* [92], *Mucor circinelloides* [93], *Mucor rouxii* [94] and *Trichosporon fermentans* [94], to name a few. Feedstock for biodiesel is traditionally vegetable oils and animal fat but the cost of these, and concerns over land use change, are prompting a search for more renewable feedstocks [95]. Some fungi are able to convert cellulose substrates into a biofuel called mycodiesel comprised of volatile organic compounds (VOCs) rather than FAMES [90], demonstrated in *Gliocladium roseum* [96], which offers the possibility of using agricultural waste, namely crop residue, as a lower cost and more sustainable feedstock.

Construction

Up to 38% of the lifetime energy demands of a typical house comes from its construction [97]; by production of materials, manufacturing processes and transport of materials [98]. Due to the growing

housing market there is a shortage of building materials such as cement, bricks, wood, steel and aluminium; many of which need huge energy input to produce and create pollution in the process [99]. Composite materials made of fungal mycelium present a possible solution for the construction industry as their production is low-energy and sustainable, the mycelium can be grown on waste substrates and the material is biodegradable [98]. Furthermore, mycelium has high tensile strength due to the presence of chitin in fungal cell walls [100], it is naturally fire-retardant [101] and is acoustically absorbent [102] so could replace wood, foam and plastics in insulation, doors, panelling and flooring [98].

Packaging

Materials made from mycelium also present a sustainable and biodegradable alternative for packaging, with the potential to replace plastics and polystyrene foams that are made from petroleum [103]. The mycelium are grown in a mould until it forms the required shape and density, then it is dehydrated to stop further growth [103]. It is now available commercially from Mushroom® Packaging who grow the mycelium from the agricultural by-product of hemp.

Fungi in bioremediation

Plastics

Commonly used synthetic plastics are polyethylene, polypropylene, nylon, polycarbonate polystyrene, polyvinylchloride (PVC), polyethylene terephthalate (PET), polytetrafluoro-ethylene (PTFE) and polyester polyurethane (PU) [104]. PU accounts for 6-7% of total plastics produced worldwide, mostly in the form of foams used in footwear, furniture and automobiles [105]. The majority of plastic waste ends up in landfill but the lack of landfill sites coupled with long-term persistence of plastics require new solutions for plastic disposal [106] and PU has been identified as vulnerable to microbial degradation, particularly by fungi [106]–[109]. In 2017, a PU-degrading fungus, identified as *Aspergillus tubingensis*, was isolated from soil at a waste disposal site in Pakistan and has the potential for large-scale biodegradation of PU and other plastic waste [104].

Crude oil

Polycyclic hydrocarbons (PAH) are a group of toxic, carcinogenic and mutagenic compounds found in crude oil that are persistent environmental pollutants, which are the focus of many studies looking at mycoremediation of contaminated soils, in particular using oyster mushrooms (*Pleurotus ostreatus*) [110]–[112]. Soils heavily contaminated with PAHs are inoculated with mycelia and within weeks the PAH content is reduced to such levels that seeds are able to germinate, which led to the Washington State of Department of Transport using the bioremediated soils from one study for highway landscaping [111]. The mushroom industry produces 5kg of spent mushroom waste (SMW) for every 1kg of mushrooms produced and this SMW from oyster mushrooms contains sufficient mycelia and nutrients for growth [113], thereby recycling a waste product for mycoremediation of polluted soils. Mycologist Paul Stamets, owner of Fungi Perfecti, is currently testing MycoBooms™, which is a floating straw arm colonized with oyster mushrooms that could be used to contain and absorb oil spills in the sea [114].

Radiation

Radionuclides are atomic nuclei that spontaneously transform or decay and, in doing so, emit energy in the form of radiation [115]. Radionuclides enter the environment naturally by interaction of cosmic rays with the atmosphere [116] but the majority come from human activity: nuclear weapons testing,

nuclear power plants, uranium mining and nuclear accidents [117]. There has been great interest in fungi as absorbents of environmental radionuclides due to the huge surface area of their hyphae [118] and their ability to grow in the presence of radiation, heavy metals and low pH [119]. For example, *Rhodotorula taiwanensis* can grow at pH 2.3 and 66 Gray per hour (Gy/h) in the presence of high concentrations of mercury and chromium [119], while *Rhizopus arrhizus* can absorb up to 180 mg of uranium per gram of its biomass [120]. Of particular interest are the fungal species found growing in the Shelter built around the damaged reactor of the Chernobyl Nuclear Power Plant [121] and wild mushrooms growing from the land around Chernobyl and Fukushima power plants, which are shown to absorb radiocaesium [122]. Accumulation of radionuclides by fungi immobilises them and prevents them leaching into soil, and mushrooms can be removed and destroyed to prevent consumption by animals or humans, hence their potential role in remediation of contaminated sites [118].

References

- [1] H. Bucking, E. Liepold, and P. Ambilwade, "The Role of the Mycorrhizal Symbiosis in Nutrient Uptake of Plants and the Regulatory Mechanisms Underlying These Transport Processes," in *InTech*, 2012, pp. 107–138.
- [2] B. Wang and Y.-L. Qiu, "Phylogenetic distribution and evolution of mycorrhizas in land plants," *Mycorrhiza*, vol. 16, pp. 299–363, 2006.
- [3] N. A. Soudzilovskaia *et al.*, "Global mycorrhizal plant distribution linked to terrestrial carbon stocks," *Nat. Commun.*, vol. 10, no. 5077, pp. 1–10, 2019.
- [4] K. Posta and N. H. Duc, "Benefits of Arbuscular Mycorrhizal Fungi Application to Crop Production under Water Scarcity," in *IntechOpen*, pp. 1–27.
- [5] P. Jeffries, S. Gianinazzi, S. Perotto, K. Turnau, and J.-M. Barea, "The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility," *Biol. Fertil. Soils*, vol. 37, pp. 1–16, 2003.
- [6] M. A. Gorzelak, A. K. Asay, B. J. Pickles, and S. W. Simard, "Inter-plant communication through mycorrhizal networks mediates complex adaptive behaviour in plant communities," *AoB Plants*, vol. 7, pp. 1–13, 2015.
- [7] B. J. Pickles, R. Wilhelm, A. K. Asay, A. S. Hahn, S. W. Simard, and W. W. Mohn, "Transfer of ¹³C between paired Douglas-fir seedlings reveals plant kinship effects and uptake of exudates by ectomycorrhizas," *New Phytol.*, vol. 214, pp. 400–411, 2017.
- [8] L. Boddy, *Fungi, Ecosystems, and Global Change*, Third Edit. Elsevier Ltd., 2016.
- [9] T. Grinhut, Y. Hadar, and Y. Chen, "Degradation and transformation of humic substances by saprotrophic fungi: processes and mechanisms," *Fungal Biol. Rev.*, vol. 21, pp. 179–189, 2007.
- [10] B. J. W. Mills, S. A. Batterman, and K. J. Field, "Nutrient acquisition by symbiotic fungi governs Palaeozoic climate transition," *Philos. Trans. B*, vol. 373, pp. 1–8, 2017.
- [11] R. Honegger, D. Edwards, L. Axe, and C. Strullu-Derrien, "Fertile Prototaxites taiti: a basal ascomycete with inoperculate, polysporous asci lacking croziers," *Philos. Trans. B*, vol. 373, pp. 1–14, 2017.
- [12] F. E. Vega *et al.*, "Fungal entomopathogens: new insights on their ecology," *Fungal Ecol.*, vol. 2, no. 4, pp. 149–159, 2009.
- [13] C. A. Deutsch *et al.*, "Increase in crop losses to insect pests in a warming climate," *Science (80-.)*, vol. 361, no. 6405, pp. 916–919, 2018.

- [14] W. Aktar, D. Sengupta, and A. Chowdhury, "Impact of pesticides use in agriculture: Their benefits and hazards," *Interdiscip. Toxicol.*, vol. 2, no. 1, pp. 1–12, 2009.
- [15] M. R. d. Faria and S. P. Wraight, "Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types," *Biol. Control*, vol. 43, no. 3, pp. 237–256, 2007.
- [16] J. Hershenhorn, F. Casella, and M. Vurro, "Weed biocontrol with fungi: past, present and future," *Biocontrol Sci. Technol.*, vol. 26, no. 10, pp. 1313–1328, 2016.
- [17] M. J. Cullen, "Bringing the cost benefits of analysis of biological control of *Chondrilla juncea* up to date.," in *Proceedings of the IV symposium on biological control of weeds*, 1985, pp. 145–152.
- [18] S. Kaewchai, K. Soyong, and K. D. Hyde, "Mycofungicides and fungal biofertilizers," *Fungal Divers.*, vol. 38, pp. 25–50, 2009.
- [19] G. Cardwell, J. F. Bornman, A. P. James, and L. J. Black, "A Review of Mushrooms as a Potential Source of Dietary Vitamin D," *Nutrients*, vol. 10, no. 1498, pp. 1–11, 2018.
- [20] A. J. Kakon, B. K. Choudhury, and S. Saha, "Mushroom is an Ideal Food Supplement," *J. Dhaka Natl. Med. Coll. Hosp.*, vol. 18, no. 01, pp. 58–62, 2012.
- [21] D. J. Royse, "A Global Perspective on the High Five: *Agaricus*, *Pleurotus*, *Lentinula*, *Auricularia* & *Flammulina*," *Proc. 8th Int. Conf. Mushroom Biol. Mushroom Prod.*, pp. 1–6, 2014.
- [22] S. Wang and M. F. Marcone, "The biochemistry and biological properties of the world's most expensive underground edible mushroom: Truffles," *Food Res. Int.*, vol. 44, no. 9, pp. 2567–2581, 2011.
- [23] C. Higgins, H. Margot, S. Warnquist, E. Obeysekare, and K. Mehta, "Mushroom Cultivation in the Developing World: A Comparison of Cultivation Technologies," *IEEE*, 2017.
- [24] T. J. A. Finnigan, B. T. Wall, P. J. Wilde, F. B. Stephens, S. L. Taylor, and M. R. Freedman, "Mycoprotein: The Future of Nutritious Nonmeat Protein, a Symposium Review," *Curr. Dev. Nutr.*, no. 4, pp. 1–5, 2002.
- [25] M. V. Copetti, "Yeasts and molds in fermented food production: an ancient bioprocess," *Curr. Opin. Food Sci.*, vol. 25, pp. 57–61, 2019.
- [26] I. N. Roberts and S. G. Oliver, "The yin and yang of yeast: biodiversity research and systems biology as complementary forces driving innovation in biotechnology," *Biotechnol. Lett.*, vol. 33, pp. 477–487, 2011.
- [27] I. of A. Studies, "Splitting the Bill: Alcohol's Impact on the Economy," 2017.
- [28] N. P. Money, *The Rise of Yeast: How the Sugar Fungus Shaped Civilisation*. 2018.
- [29] S. Dashko, N. Zhou, C. Compagno, and J. Piskur, "Why, when, and how did yeast evolve alcoholic fermentation?," *FEMS Yeast Res.*, vol. 14, pp. 826–832, 2014.
- [30] Y. Zhu and J. Tramper, "Koji – where East meets West in fermentation," *Biotechnol. Adv.*, vol. 31, no. 8, pp. 1448–1457, 2013.
- [31] I. Franciosa, V. Alessandria, P. Dolci, K. Rantsiou, and L. Cocolin, "Sausage fermentation and starter cultures in the era of molecular biology methods," *Int. J. Food Microbiol.*, vol. 279, pp. 26–32, 2018.
- [32] A. Fleming, "Classics in infectious diseases: on the antibacterial action of cultures of a

- penicillium, with special reference to their use in the isolation of *B. influenzae*," *Br. J. Exp. Pathol.*, vol. 10, pp. 226–236, 1929.
- [33] S. Y. Tan and Y. Tatsumura, "Alexander Fleming (1881–1955): Discoverer of penicillin," *Singapore Med. J.*, vol. 56, no. 7, pp. 366–367, 2015.
 - [34] E. P. Abraham, G. G. Newton, K. Crawford, H. S. Burton, and C. W. Hale, "Cephalosporin N: a new type of penicillin," *Nature*, vol. 171, no. 4347, p. 343, 1953.
 - [35] J. Silber, A. Kramer, A. Labes, and D. Tasdemir, "From Discovery to Production : Biotechnology of Marine Fungi for the Production of New Antibiotics," *Mar. Drugs*, vol. 14, no. 137, pp. 1–20, 2016.
 - [36] K. Kinoshita, "Über eine neue Aspergillus Art, *A. itaconicus*," *Bot. Mag. Tokyo*, vol. 45, pp. 45–50, 1931.
 - [37] R. Hanson and T. E. Elbe, "No Title," *J. Bacteriol.*, vol. 58, p. 527, 1949.
 - [38] W. O. Godtfredsen, S. Jahnsen, H. Lorck, K. Roholt, and L. Tybring, "Fusidic Acid: a New Antibiotic," *Nature*, vol. 4819, p. 987, 1962.
 - [39] C. E. Meyer and F. Reusser, "A Polypeptide Antibacterial Agent Isolated from *Trichoderma viride*," *Experientia*, vol. 23, no. 2, pp. 85–86, 1967.
 - [40] R. A. Bucknall, H. Moores, R. Simms, and B. Hesp, "Antiviral Effects of Aphidicolin, a New Antibiotic Produced by *Cephalosporium aphidicola*," *Antimicrob. Agents Chemother.*, vol. 4, no. 3, pp. 294–298, 1973.
 - [41] S. Choudhury, M. P. Rana, T. K. Chatterjee, U. K. Mazumder, and M. Gupta, "Antimicrobial activities of mycotoxin MT81 and its structural derivatives," *Indian J. Exp. Biol.*, vol. 30, no. 2, pp. 140–1, 1992.
 - [42] M. Tanaka, T. Fukushima, Y. Tsujino, and T. Fujimori, "Nigrosporins A and B, New Phytotoxic and Antibacterial Metabolites Produced by a Fungus *Nigrospora oryzae*," *Biosci. Biotechnol. Biochem.*, vol. 61, no. 11, pp. 1848–1852, 1997.
 - [43] A. E. Oxford, H. Raistrick, and P. Simonart, "Griseofulvin, C₁₇H₁₇O₆Cl, a Metabolic Product of *Penicillium griseo-fulvum* Dierckx," pp. 240–248, 1938.
 - [44] T. Anke, F. Oberwinkler, W. Steglich, and G. Schramm, "The Strobilurins - New Antifungal Antibiotics from the Basidiomycete *Strobilurus tenacellus*," *J. Antibiot. (Tokyo)*, pp. 806–810, 1977.
 - [45] R. Nyfeler and W. Keller-Schierlein, "Metabolites of microorganisms. 143. Echinocandin B, a novel polypeptide-antibiotic from *Aspergillus nidulans* var. *echinulatus*: isolation and structural components," *Helv. Chim. Acta*, vol. 57, no. 8, pp. 2459–77, 1974.
 - [46] P. Traxler, J. Gruner, and J. A. L. Auden, "Papulacandins, a New Family of Antibiotics with Antifungal Activity," *J. Antibiot. (Tokyo)*, vol. XXX, no. 4, pp. 289–296, 1977.
 - [47] G. Tamura, K. Ando, S. Suzuki, A. Takatsuki, and K. Arima, "Antiviral Activity of Brefeldin A and Verrucaric Acid," *J. Antibiot. (Tokyo)*, pp. 160–161, 1968.
 - [48] G. Ma *et al.*, "Antimicrobial and Antileishmanial Activities of Hypocrellins A and B," *Antimicrob. Agents Chemother.*, vol. 48, no. 11, pp. 4450–4452, 2004.
 - [49] M. Iwatsuki, S. Takada, M. Mori, A. Ishiyama, M. Namatame, and A. Nishihara-tsukashima, "In vitro and in vivo antimalarial activity of puberulic acid and its new analogs , viticolins A–C ,

- produced by *Penicillium* sp. FKI-4410," *J. Antibiot. (Tokyo)*, vol. 64, no. November 2010, pp. 183–188, 2011.
- [50] S. S. S. S. Group, "Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S)," *Lancet*, vol. 344, no. November 19, pp. 1383–1389, 1994.
 - [51] J. Shepherd *et al.*, "Prevention of Coronary Heart Disease with Pravastatin in Men with Hypercholesterolemia," *N. Engl. J. Med.*, vol. 333, no. 20, pp. 1301–1307, 1995.
 - [52] P. H. C. S. Group, "MRC / BHF Heart Protection Study of cholesterol lowering with simvastatin in 20 536 high-risk individuals : a randomised placebo- controlled trial," *Lancet*, vol. 360, pp. 7–22, 2002.
 - [53] A. Endo, M. Kuroda, and K. Tanzawa, "Competitive Inhibition of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase by ML-236A and ML-236B Fungal Metabolites, having Hypocholesterolemic Activity," *FEBS Lett.*, vol. 72, no. 2, pp. 3–6, 1976.
 - [54] S. Brown, J. R. Faust, and J. L. Goldstein, "Induction of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Human Fibroblasts Incubated with Compactin (ML-236B), a Competitive Inhibitor of the Reductase," *J. Biol. Chem.*, vol. 253, no. 4, pp. 1121–1128, 1978.
 - [55] M. Manzoni and M. Rollini, "Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs," *Appl. Microb. Biotechnol.*, vol. 58, pp. 555–564, 2002.
 - [56] K. C. L. Mulder, F. Mulinari, O. L. Franco, M. S. F. Soares, B. S. Magalhães, and N. S. Parachin, "Lovastatin production: From molecular basis to industrial process optimization," *Biotechnol. Adv.*, vol. 33, no. 6, pp. 648–665, 2015.
 - [57] R. L. Carhart-harris *et al.*, "Psilocybin for treatment-resistant depression: fMRI-measured brain mechanisms," *Sci. Rep.*, no. June, pp. 1–11, 2017.
 - [58] C. R. McKay, A. L. Halberstadt, and G. R. Greer, "Pilot Study of Psilocybin Treatment for Anxiety in Patients With Advanced-Stage Cancer," vol. 68, no. 1, pp. 71–78, 2020.
 - [59] M. W. Johnson, A. Garcia-romeu, M. P. Cosimano, and R. R. Griffiths, "Pilot study of the 5-HT_{2A}R agonist psilocybin in the treatment of tobacco addiction," *J. Psychopharmacol.*, vol. 28, no. 11, pp. 983–992, 2014.
 - [60] M. P. Bogenschütz, A. A. Forcehimes, J. A. Pommy, C. E. Wilcox, P. C. R. Barbosa, and R. J. Strassman, "Psilocybin-assisted treatment for alcohol dependence: a proof-of-concept study," *J. Psychopharmacol.*, vol. 29, no. 3, pp. 289–299, 2015.
 - [61] F. A. Moreno, C. B. Wiegand, E. K. Taitano, and P. L. Delgado, "Safety, Tolerability, and Efficacy of Psilocybin in 9 Patients With Obsessive-Compulsive Disorder," *J. Clin. Psychiatry*, vol. 67, no. 11, pp. 1735–1740, 2006.
 - [62] M. Osmak, "Statins and cancer: current and future prospects," *Cancer Lett.*, vol. 324, no. 1, pp. 1–12, 2012.
 - [63] M. Moradali, H. Mostafavi, S. Ghods, and G. Hedjaroude, "Immunomodulating and anticancer agents in the realm of macromycetes fungi (macrofungi)," *Int. Immunopharmacol.*, vol. 7, pp. 701–724, 2007.
 - [64] A.-T. Yap and M.-L. Ng, "Immunopotentiating Properties of Lentinan (1-3)- β -D-glucan Extracted from Culinary–Medicinal Shiitake Mushroom *Lentinus edodes*," *Int. J. Med. Mushrooms*, vol. 5, no. 4, 2003.

- [65] J. M. Bryant, M. Bouchard, and A. Haque, "Anticancer Activity of Ganoderic Acid DM: Current Status and Future Perspective," *J. Clin. Cell. Immunol.*, vol. 8, no. 6, pp. 1–15, 2018.
- [66] J. W. Bok, L. Lerner, J. Chilton, H. G. Klingeman, and G. H. N. Towers, "Antitumor sterols from the mycelia of *Cordyceps sinensis*," *Phytochemistry*, vol. 51, pp. 891–898, 1999.
- [67] S. Luk *et al.*, "Chemopreventive Effect of PSP Through Targeting of Prostate Cancer Stem Cell-Like Population," *PLoS One*, vol. 6, no. 5, pp. 1–9, 2011.
- [68] A. Kornienko *et al.*, "Towards a Cancer Drug of Fungal Origin," *Med. Res. Rev.*, vol. 35, no. 5, pp. 937–967, 2016.
- [69] H. Tribe, "The Discovery and Development of Cyclosporin," *Mycologist*, vol. 12, no. 1, pp. 20–22, 1998.
- [70] H. Ishikawa, "Mizoribine and mycophenolate mofetil," *Curr. Med. Chem.*, vol. 6, no. 7, pp. 575–597, 1999.
- [71] T. B. Regueira, K. R. Kildegaard, B. G. Hansen, U. H. Mortensen, C. Hertweck, and J. Nielsen, "Molecular Basis for Mycophenolic Acid Biosynthesis in *Penicillium brevicompactum*," *Appl. Environ. Microbiol.*, vol. 77, no. 9, pp. 3035–3043, 2011.
- [72] C. Julie, E. Lobkovsky, N. B. Pliam, G. Strobel, and J. Clardy, "Subglutinols A and B: Immunosuppressive compounds from the endophytic fungus *Fusarium subglutinans*," *J. Org. Chem.*, vol. 60, no. 1, pp. 7076–7077, 1995.
- [73] D. Martinez *et al.*, "Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei*," *Nat. Biotechnol.*, vol. 26, no. 5, pp. 553–561, 2008.
- [74] A. Miettinen-Oinonen, "Chapter 4: Cellulases in the Textile Industry," in *Industrial Enzymes*, 2007, pp. 51–63.
- [75] L. Heikinheimo, J. Buchert, A. Miettinen-Oinonen, and P. Suominen, "Treating Denim Fabrics with *Trichoderma Reesei* Cellulases," *Text. Res. J.*, vol. 70, no. 11, pp. 969–973, 2000.
- [76] A. M. Amorim, M. D. G. Gasques, J. Andreus, and M. Scharf, "The application of catalase for the elimination of hydrogen peroxide residues after bleaching of cotton fabrics," *Ann. Brazilian Acad. Sci.*, vol. 74, no. 3, pp. 433–436, 2002.
- [77] A. Nema, S. H. Patnala, V. Mandari, S. Kota, and S. K. Devarai, "Production and optimization of lipase using *Aspergillus niger* MTCC 872 by solid-state fermentation," *Bull. Natl. Res. Cent.*, vol. 43, no. 82, pp. 1–8, 2019.
- [78] F. R. De Souza and M. Gutterres, "Applications of Enzymes in Leather Processing: a Comparison between Chemical and Coenzymatic Processes," *Brazilian J. Chem. Eng.*, vol. 29, no. 03, pp. 473–481, 2012.
- [79] F. Hasan, A. A. Shah, S. Javed, and A. Hameed, "Enzymes used in detergents: Lipases," *African J. Biotechnol.*, vol. 9, no. 31, pp. 4836–4844, 2010.
- [80] D. De Santis, M. Moresi, A. M. Gallo, and M. Petruccioli, "Assessment of the dyeing properties of pigments from *Monascus purpureus*," *J. Chem. Technol. Biotechnol.*, vol. 80, pp. 1072–1079, 2005.
- [81] V. A. Hernández, F. Galleguillos, R. Thibaut, and A. Müller, "Fungal dyes for textile applications: testing of industrial conditions for wool fabrics dyeing," *J. Text. Inst.*, vol. 110, no. 1, pp. 61–66, 2019.

- [82] R. Raisanen, P. Nousiainen, and P. H. Hynninen, "Emodin and Dermocybin Natural Anthraquinones as Mordant Dyes," *Text. Res. J.*, vol. 71, no. 11, pp. 1016–1022, 2001.
- [83] FAO, "Global Forest Products: Facts and Figures," 2018.
- [84] C. E. Torres, C. Negro, E. Fuente, and A. Blanco, "Enzymatic approaches in paper industry for pulp refining and biofilm control," *Appl. Microbiol. Biotechnol.*, vol. 96, pp. 327–344, 2012.
- [85] A. P. M. Tavares, A. M. R. B. Xavier, and D. V. Evtuguin, "Biotechnology Applications in Pulp and Paper Industry," *Biotechnology*, vol. 12, pp. 561–581, 2014.
- [86] P. Bajpai, "Application of Enzymes in the Pulp and Paper Industry," *Biotechnol. Prog.*, vol. 15, pp. 147–157, 1999.
- [87] A. A. El-Imam and C. Du, "Fermentative Itaconic Acid Production," *J. Biodiversity, Bioprospecting Dev.*, vol. 1, no. 1, pp. 1–8, 2014.
- [88] M. Okabe, D. Lies, S. Kanamasa, and E. Y. Park, "Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus*," *Appl. Microbiol. Biotechnol.*, vol. 84, pp. 597–606, 2009.
- [89] S. Kristiansen, "The journey of developing sustainable materials for LEGO bricks."
- [90] P. R. Babu and V. V. Sarma, "Chapter 11 - Fungi as Promising Biofuel Resource," in *New and Future Developments in Microbial Biotechnology and Bioengineering*, 2019, pp. 149–164.
- [91] H. Gema, A. Kavadia, D. Dimou, V. Tsagou, M. Komaitis, and G. Aggelis, "Production of γ -linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel," *Appl. Microbiol. Biotechnol.*, vol. 58, pp. 303–307, 2002.
- [92] V. K. Eroshin, A. D. Satroutdinov, E. G. Dedyukhina, and T. I. Chistyakova, "Arachidonic acid production by *Mortierella alpina* with growth-coupled lipid synthesis," *Process Biochem.*, vol. 35, pp. 1171–1175, 2000.
- [93] G. Vicente *et al.*, "Direct Transformation of Fungal Biomass from Submerged Cultures into Biodiesel," *Energy & Fuels*, vol. 24, pp. 3173–3178, 2010.
- [94] S. S. Mamatha, R. Ravi, and G. Venkateswaran, "Medium Optimization of Gamma Linolenic Acid Production in *Mucor rouxii* CFR -G15 using RSM," *Food Bioprocess Technol.*, vol. 1, pp. 405–409, 2008.
- [95] Y. Zheng, X. Yu, J. Zeng, and S. Chen, "Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw," *Biotechnol. Biofuels*, vol. 5, no. 50, pp. 1–10, 2012.
- [96] G. A. Strobel *et al.*, "The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL 50072)," *Microbiology*, vol. 154, pp. 3319–3328, 2008.
- [97] I. Sartori and A. G. Hestnes, "Energy use in the life cycle of conventional and low-energy buildings : A review article," *Energy Build.*, vol. 39, pp. 249–257, 2007.
- [98] M. Jones, A. Mautner, S. Luenco, A. Bismarck, and S. John, "Engineered mycelium composite construction materials from fungal biorefineries: A critical review," *Mater. Des.*, vol. 187, pp. 1–16, 2020.
- [99] M. V. Madurwar, R. V. Ralegaonkar, and S. A. Mandavgane, "Application of agro-waste for sustainable construction materials: A review," *Constr. Build. Mater.*, vol. 38, pp. 872–878,

- 2013.
- [100] M. Nakajima, K. Atsumi, K. Kifune, K. Mjura, and H. Kanamaru, "Chitin is an Effective Material for Sutures," *Jpn. J. Surg.*, vol. 16, no. 6, pp. 418–424, 1986.
 - [101] M. Jones *et al.*, "Thermal Degradation and Fire Properties of Fungal Mycelium and Mycelium - Biomass Composite Materials," *Sci. Rep.*, vol. 8, no. 17583, pp. 1–10, 2018.
 - [102] M. G. Pelletier, G. A. Holt, J. D. Wanjura, E. Bayer, and G. McIntyre, "An evaluation study of mycelium based acoustic absorbers grown on agricultural by-product substrates," *Ind. Crops Prod.*, vol. 51, no. November, pp. 480–485, 2013.
 - [103] R. Abhijith, A. Ashok, and C. R. Rejeesh, "Sustainable packaging applications from mycelium to substitute polystyrene: a review," *Mater. Today Proc.*, vol. 5, pp. 2139–2145, 2018.
 - [104] S. Khan *et al.*, "Biodegradation of polyester polyurethane by *Aspergillus tubingensis*," *Environ. Pollut.*, vol. 225, pp. 469–480, 2017.
 - [105] M. Cregut, M. Bedas, M. Durand, and G. Thouand, "New insights into polyurethane biodegradation and realistic prospects for the development of a sustainable waste recycling process," *Biotechnol. Adv.*, vol. 31, no. 8, pp. 1634–1647, 2013.
 - [106] U. Zafar, A. Houlden, and G. D. Robson, "Fungal Communities Associated with the Biodegradation of Polyester Polyurethane Buried under Compost at Different Temperatures," *Appl. Environ. Microbiol.*, vol. 79, no. 23, pp. 7313–7324, 2013.
 - [107] J. Álvarez-Barragán, L. Domínguez-Malfavón, M. Vargas-Suárez, R. González-Hernández, G. Aguilar-Osorio, and H. Loza-Tavera, "Biodegradative Activities of Selected Environmental Fungi on a Polyester Polyurethane Varnish and Polyether Polyurethane Foams," *Appl. Environ. Microbiol.*, vol. 82, no. 17, pp. 5225–5235, 2016.
 - [108] S. R. Barratt, A. R. Ennos, M. Greenhalgh, G. D. Robson, and P. S. Handley, "Fungi are the predominant micro-organisms responsible for degradation of soil-buried polyester polyurethane over a range of soil water holding capacities," *J. Appl. Microbiol.*, vol. 95, pp. 78–85, 2003.
 - [109] J. R. Russell *et al.*, "Biodegradation of Polyester Polyurethane by Endophytic Fungi," *Appl. Environ. Microbiol.*, vol. 77, no. 17, pp. 6076–6084, 2011.
 - [110] M. Biiatt, T. Cajthami, and V. Sasek, "Mycoremediation of PAH-Contaminated Soil," *Folia Microbiol. (Praha)*, vol. 47, no. 3, pp. 255–258, 2002.
 - [111] S. Thomas, P. Becker, M. R. Pinza, and J. Q. Word, "Mycoremediation of Aged Petroleum Hydrocarbon Contaminants in Soil," 1998.
 - [112] S. Adams, Q. Ak, and K. Stenchly, "Mycoremediation of Diesel Contaminated Soil with Oyster Mushroom (*Pleurotus ostreatus*) using Maize (*Zea mays*) as the Test Crop," *UDS Int. J. Dev.*, vol. 2, no. 2, pp. 1–8, 2015.
 - [113] K. L. Lau, Y. Y. Tsang, and S. W. Chiu, "Use of spent mushroom compost to bioremediate PAH-contaminated samples," *Chemosphere*, vol. 52, pp. 1539–1546, 2003.
 - [114] P. Stamets, "The Petroleum Problem," 2010. [Online]. Available: <https://fungi.com/blogs/articles/the-petroleum-problem>.
 - [115] C. Keller, W. Wolf, and J. Shani, "Radionuclides, 2. Radioactive Elements and Artificial Radionuclides," *Ullmann's Encycl. Ind. Chem.*, vol. 31, 2012.

- [116] R. W. Perkins and J. M. Nielsen, "Cosmic-Ray Produced Radionuclides in the Environment," *Health Phys.*, vol. 11, pp. 1297–1304, 1965.
- [117] Q. Hu, J. Weng, and J. Wang, "Sources of anthropogenic radionuclides in the environment: a review," *J. Environ. Radioact.*, vol. 101, no. 6, pp. 426–437, 2010.
- [118] J. Dighton, T. Tugay, and N. Zhdanova, "Fungi and ionizing radiation from radionuclides," *FEMS Microbiol. Lett.*, vol. 281, pp. 109–120, 2008.
- [119] R. Tkavc *et al.*, "Prospects for Fungal Bioremediation of Acidic Radioactive Waste Sites: Characterization and Genome Sequence of *Rhodotorula taiwanensis* MD1149," *Front. Microbiol.*, vol. 8, no. 2538, pp. 1–21, 2018.
- [120] J. M. Tobin, D. G. Cooper, and R. J. Neufeld, "Uptake of Metal Ions by *Rhizopus arrhizus* Biomass," *Appl. Environ. Microbiol.*, vol. 47, no. 4, pp. 821–824, 1984.
- [121] N. N. Zhdanova, V. A. Zakharchenko, V. V Vember, and L. T. Nakonechnaya, "Fungi from Chernobyl: mycobiota of the inner regions of the containment structures of the damaged nuclear reactor," *Mycol. Res.*, vol. 104, pp. 1421–1426, 2000.
- [122] T. Ohnuki *et al.*, "Role of filamentous fungi in migration of radioactive cesium in the Fukushima forest soil environment," *Environ. Sci. Process. Impacts*, vol. 21, pp. 1164–1173, 2019.

Appendix 3: Google sign-up form for the first air sampling round that was linked to by a shortened URL on the poster and in all recruitment Tweets, Facebook posts and emails.

Questions

Responses

505



Science Solstice

A citizen science project taking place on Thursday 21st June (Summer Solstice 2018) involving UK-wide passive outdoor sampling of fungal spores for monitoring of azole fungicide drug resistance. All you need to do is place your air samplers on groundfloor outdoor windowsills at your two chosen locations (e.g. home and workplace) and leave for ~8 hours. Return them, along with the completed questionnaire, in the freepost envelope at your earliest convenience.

You will receive a package containing 2 x air samplers, a poster, instructions, information page, questionnaire and freepost return envelope in the fortnight leading up to Summer Solstice and a reminder text the day before. If you have not received the air sampling pack by Monday 18th June please email j.shelton@imperial.ac.uk and I will repost.

GDPR compliance: All personal details collected in this form will be kept confidential and data points will be anonymised. Your name and address are required for posting the air sampling package to you: after posting, these details will be deleted and only the locations you provide on the completed questionnaire will be assigned to samples for spatial analyses. Your mobile number is required to send you a text the night before Summer Solstice to remind you of experiment day. Providing your email address is optional and will be taken as consent to contact you with project updates and a link to the online interactive map to see your anonymised data points; please leave blank if you would prefer not to be contacted. At no point will your data be shared with any third party and it will only be kept on record for the duration of the project.

If at any time you wish to opt out of this project and for your data to be removed from the database, please email j.shelton@imperial.ac.uk.

More details can be found on www.fisherlab.co.uk/blog/science-solstice or on Twitter by following @jenmgshe and #ScienceSolstice.

Name *

Short answer text

Address *

Long answer text

Mobile number *

Short answer text

Email address (optional)

Short answer text

Which research institute do you belong to? (If any; for combined postage)

- ☐ CEH - Bangor
- ☐ CEH - Edinburgh
- ☐ CEH - Lancaster
- ☐ CEH - Wallingford
- ☐ Imperial College London - South Ken campus
- ☐ Imperial College London - St Mary's campus
- ☐ Other...

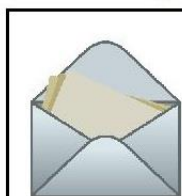
How did you hear about this project?

- ☐ Email
- ☐ Facebook
- ☐ Poster
- ☐ Twitter
- ☐ Word-of-mouth
- ☐ Other...

Appendix 4: Instructions explaining when and how to expose the air samplers to participate in the citizen science air sampling project. These instructions were for initial air sampling round on 21st June 2018 and the date was amended for subsequent sampling rounds.



A SIMPLE GUIDE TO: Passive outdoor air sampling



Your air sampling pack should contain:

- Instructions (this!)
- Poster (put it up to remind you!)
- Information page
- 2 x air sampler + Blu Tack
- Freepost return envelope



On **Thursday 21st June** (a.k.a. Summer Solstice 2018) it's experiment time!

Follow the instructions below to collect your passive air samples.

IMPORTANT NOTE! If there is torrential rain on Thursday 21st June this air sampling method will not work! Please do the experiment at your two chosen locations on the soonest dry day and specify the date on your questionnaire.



1. Identify an outdoor ground floor windowsill at your home and workplace.

2. Orientate the air sampler so the white label is on top and clear side with Blu Tack is facing down (see right).



3. Secure air sampler to windowsill using Blu Tack.



4. Peel off (and retain) white label and leave air sampler, sticky side up, for 6-8 hours.



5. Reattach white label and post when possible.

When your sampling is finished please place your two air samplers inside the freepost return envelope, along with your completed questionnaire, and store in a cool and dark place until you are able to return to me by post.

Appendix 5: Questionnaire to be completed and returned in the Freepost envelope detailing the date air samples were collected and the exact locations where the air samplers were placed and exposed. Participants were asked to provide an email address if they wished to receive future project updates.



SCIENCE SOLSTICE QUESTIONNAIRE



(Please complete and return with the air samplers in the freepost envelope.)

Name: _____

Date collected: _____

LOCATION 1 (e.g. home)

Address: _____

Or GPS coordinates: (latitude) _____ (longitude) _____

Outdoor ground floor windowsill: Y/N

If no, please describe location: _____

LOCATION 2 (e.g. workplace)

Address: _____

Or GPS coordinates: (latitude) _____ (longitude) _____

Outdoor ground floor windowsill: Y/N

If no, please describe location: _____

Thank you for taking part in this Science Solstice air sampling project! If you would like to receive project updates and a link to the online interactive map to view your anonymized data points, please provide your email address:

Appendix 6: A blog post written about the air sampling citizen science project was published online when recruitment began, and a paper copy was included in each sampling pack.

#ScienceSolstice – citizen science meets microbiology!

What is this project about?

Thank you for your interest in my upcoming citizen science project! This is part of my PhD project titled “Environmental drivers of azole-resistance in *Aspergillus fumigatus* and its relevance to human health” split between Professor Mat Fisher’s group at Imperial College London and Dr Andrew Singer’s group at the Centre for Ecology and Hydrology. For my project I am conducting a UK-wide survey to determine background levels of mould spores (*Aspergillus fumigatus*) by collecting air and soil samples from as many places as possible, and then testing them for resistance to azole fungicide drugs.

This is where you come in! By agreeing to participate in my Science Solstice passive air sampling experiment you will be providing me with valuable data points for my project. All you need to do is leave the air samplers sticky side up on an outside windowsill on the ground floor of two different locations (e.g. your house and workplace) for the whole day of **Thursday 21st June** and then freepost them back to me in the envelope provided. As your air samplers sit on the windowsills the fungal spores passing through the air around them will settle and stick to them, making this a passive sampling method (as opposed to actively pumping large volumes of air through a filter to capture fungal spores).

How do I get involved?

If you would like to take part please enter your details in the Google form: <https://tinyurl.com/sciencesolstice>. **Your details will be kept completely confidential.** If you choose to provide an email address, I will send you updates on the project and a link to your anonymized data points on an online interactive map. (If you are happy to take part but would not like further updates please leave the email space blank and I will not trouble you further!) In the fortnight prior to Summer Solstice you will receive an envelope containing the air samplers, simple instructions, a questionnaire and a freepost envelope- all you need to complete the experiment- and you will receive a text message reminder the day before so you don’t forget. Thank you in advance for your participation!



Online interactive map for logging Science Solstice data points, courtesy of EpiCollect (<http://www.epicollect.net/>).

Talk science to me!

Aspergillus fumigatus plays an important role in the environment as a decomposer- it is commonly found in soil and decaying vegetation where it breaks down organic matter into carbon and nitrogen- and it produces tiny spores that readily become airborne. These spores spread easily on wind currents meaning that *A. fumigatus* is EVERYWHERE. It is found in most countries, outdoors and indoors, in air and in soil, and it is estimated that we each inhale 100s of spore every day. When we are healthy these spores are cleared by our innate immune system and cause us no problems, whereas in individuals with a compromised immune response- such as those with HIV/AIDS or cystic fibrosis or undergoing organ transplant or chemotherapy- these spores can establish and grow in the lungs to cause invasive aspergillosis (IA). The first line of treatment of IA is with drugs containing active compounds called azoles and the fatality rate varies between 40-90%, dependent on the patient's history. Unfortunately, if the patient is infected with *A. fumigatus* that has developed resistance to these medical azoles the infection becomes much harder to treat and the fatality rate is higher. Azoles used in medical drugs are structurally similar to azoles incorporated into fungicide sprays for agricultural use and it is likely that *A. fumigatus* in the soil that is accidentally exposed to fungicides (it is not the intended target as it is not a crop pathogen) can develop resistance to azoles- both medical and agricultural. By collecting air and soil samples from across the UK I will determine the percentage of spores that have developed azole resistance, and whether this is influenced by factors such as seasonality, land use, soil type and urban versus rural. These data could then inform an environmental "risk map" for individuals at highest risk of acquiring IA.



A. fumigatus colonies grown from sticky tape used in my pilot study at Imperial's St Mary's campus.

Appendix 7: Blog post about soil sampling citizen science project published on UKCEH website.



UK Centre for
Ecology & Hydrology

[About us](#) [Science](#) [Data](#) [Services](#) [Staff](#) [News & media](#) [Cymraeg](#) 

Summer Solstice Kickoff

Submitted by **Dr. Andrew C. Singer** on Mon, 10/06/2019 - 14:32

**** Sign-ups have now closed for the Summer Solstice citizen science project. Thanks to everyone for the interest shown in taking part. ****

Hot on the heels of a citizen science project that had hundreds of people placing air samplers on their window sills, Jenny Shelton, a PhD student with the Centre for Ecology & Hydrology (CEH) and Imperial College, has sought help again as part of her research into monitoring fungicide drug resistance. Her new survey is dubbed #SummerSolstice and asks volunteers to send in soil or compost samples taken from their gardens on the Summer Solstice (June 21).

Jenny's supervisors, Andrew Singer of CEH and Mat Fisher of Imperial College, explain why the science is so important:

The new survey is a follow-on from Jenny's **#ScienceSolstice** project, which looked for azole-resistant *Aspergillus fumigatus* in samples of air collected from the homes and workplaces of citizen scientists on solstice and equinox days during 2018-19. *A. fumigatus* is a ubiquitous fungal species commonly found in soil due to its important role as a decomposer but which is also linked to a disease called aspergillosis or 'fungal asthma'.

The spores pose no risk to most people as they are cleared by our bodies' immune system, but some individuals suffer debilitating allergic responses caused by hypersensitisation to the inhaled spores.

If spores are not cleared they can germinate in the lung where the growing fungus secretes toxins and triggers an immune response, leading to symptoms such as weight loss, chronic cough and breathlessness, and resulting in a diagnosis of aspergillosis. In individuals with a compromised immune response- such as those with HIV/AIDS or cystic fibrosis or undergoing organ transplant or chemotherapy- spores may enter the bloodstream via the lungs and disseminate through the body, resulting in a life-threatening illness called invasive aspergillosis (IA).



SUMMER SOIL-STICE
Friday 21st June 2019

**** BE A CITIZEN SCIENTIST FOR THE DAY! ****
Join fellow citizen scientists across the U.K. in sampling your garden soil for fungal spores, to monitor for resistance to azole fungicide drugs

Imperial College London | Centre for Ecology & Hydrology | NERC

SHARE THIS PAGE

[f](#) [t](#) [y](#) [in](#)

Recent Events

 **About Drought Download**
2019-11-07

...

 **Shaping the Development of the CEH UK-SCAPE Data Science Framework**
2019-11-12

CEH is developing a...

Recent Publications

Metal speciation from stream to open ocean: modelling v. measurement

Analysis of 129I and 127I in soils of the Chernobyl Exclusion Zone, 29 years after the deposition of 129I

Moving radiation protection on from the limitations of empirical concentration ratios

Aspergillosis and IA are treated with drugs containing azole compounds but infections are much harder to treat if caused by azole-resistant *Aspergillus fumigatus* spores. As the structure of the azole compounds used in medical drugs are similar to azoles used in fungicide sprays in agriculture and horticulture, it is possible that spores are acquiring resistance in the environment prior to being inhaled as well as in the lung as a result of long-term treatment.

Participation from across the UK

The idea behind the citizen science projects is to get wide participation from across the UK in order to determine the background levels of azole-resistant *A. fumigatus* spores in air and soil and therefore get an idea of the exposure of people to these spores as we go about our daily routines.

For this phase, we're asking volunteers to send in soil/compost samples from their gardens so that Jenny can culture spores from *A. fumigatus* and then test them for fungicide resistance. As with the air sampling, knowing the location and timing of each sample will allow Jenny to look for clusters of resistance and any associations with land use, soil type, urban versus rural location and seasonality, which could identify potential drivers of resistance and potential hotspots for the drug-resistant fungus.

"...knowing the location and timing of each sample will allow Jenny to look for clusters of resistance and any associations with land use, soil type, urban versus rural location and seasonality..."

We know that industrial composters constitute a 'hotspot' for *A. fumigatus* growth because the heat generated in the centre of the waste piles during the composting process kills most bacteria and fungi but allows the thermophilic (heat-loving) *A. fumigatus* to thrive.

These facilities accept organic waste from households, restaurants and shops and also from plant nurseries, landscaping companies and farms, from which the plant matter may have been exposed to azole-containing fungicides during its lifetime. An important early stage of the composting process is digestion of the organic matter by microbes that require oxygen. The waste piles are rotated to allow for the circulation of air, thereby mixing the contents and exposing more to azole residues from the commercial and agricultural waste. This practice makes it more likely that *A. fumigatus* proliferating in the centre of the piles is exposed to azoles during its growth, which might cause it to develop resistance.

It's possible that the compost heaps we keep in our gardens act in a similar manner but on a smaller scale, and that the azole residues that drive resistance are coming from the plants and bulbs we have planted in our garden that have already been sprayed with or dipped in azoles at some point during their lifetime. Many industrial composters supply compost to farms and garden centres so it's also possible that the bags of compost we use in our gardens already contain azole-resistant *A. fumigatus* spores as a result of the composting process.

Who shouldn't take part

Jenny would like as many citizen scientists as possible to take part in the project, but it's important to recognise that compost and compost heaps potentially contain high numbers of *A. fumigatus* that may be aerosolised as a result of disturbing the bag or heap ([read more](#)). Due to this, please **do not participate** in sample collection from these locations if you suffer from aspergillosis, have a lung condition (chronic or acute, such as 'flu) or are immunosuppressed, all of which places you at greater risk of contracting aspergillosis from inhaling a large number of spores.

How you can take part

Sign-ups for the sampling packs have now closed. Thanks to the hundreds of you who have volunteered to take part, and apologies to those of you unable to apply.

The sampling pack will contain two clear plastic sachets, a wooden spatula, a poster, simple instructions and a questionnaire and will arrive prior to Friday 21 June (summer solstice 2019).

All you need to do is collect two soil samples from your garden – either from a compost heap, bag of compost or manure, pot, planter or border – and return them in the freepost envelope along with a completed questionnaire.

Jenny will process the soil samples in the lab and culture from them any *A. fumigatus* spores present, which she will then test for resistance to tebuconazole (the third most-sprayed azole in agricultural fungicides here in the UK). Those of you who indicate you are happy to receive updates will later receive an email with a link to a Google map showing anonymised locations of the samples and how many spores were present in each.

Follow Jenny on Twitter [@jenmgshe](#) to keep up on the progress of [#SummerSoilstice!](#)

Supervisory team:

Dr Andrew Singer	Prof Matthew Fisher
Senior Scientist	Faculty of Medicine, School of Public Health
Centre for Ecology & Hydrology	Imperial College London, UK
acsi @ ceh.ac.uk	matthew.fisher @ imperial.ac.uk
@OxonAndrew	@fisher_mat

Appendix 8: Instructions explaining how to collect soil samples to participate in the citizen science experiment.



A SIMPLE GUIDE TO: Outdoor soil sampling

**Imperial College
London**

 **Centre for
Ecology & Hydrology**



Your soil sampling pack should contain:

- Instructions (this!)
- Poster (put it up to remind you!)
- Information page
- 2 x clear plastic sachets
- 1 x wooden spatula
- Freepost return envelope



On Friday 21st – Sunday 23rd June
(weekend of Summer Solstice 2019)
it's experiment time!

Follow the instructions below to
collect your outdoor soil samples.

1. Identify two locations with soil, compost or rotting plant material at your home or workplace. For example:

☐ pot/planter

☐ border

☐ bag of compost

☐ compost heap



!!! Please exercise caution if sampling from a bag of compost or compost heap as disturbing the contents may cause a plume of fungal spores to be released. Collect from the top or side without moving bag or heap.

2. Unseal the clear plastic sachet, lay on the soil, squeeze from the sides to open and use the wooden spatula to scrape soil, compost or rotting plant material into the sachet.



3. Fill the sachet until loosely packed with soil, but not bulging (to prevent sachet splitting in transit!).

When your sampling is finished please place your two sachets of soil inside the freepost return envelope, along with your completed questionnaire and wooden spatula (to keep envelope flat), and store in a cool and dark place until you are able to return to me by post.

Appendix 9: Questionnaire to be completed for the soil sampling project and returned in the Freepost envelope detailing the date sample collected, geographical location of the garden that soils were collected from, type of sample collected and sample details.



SUMMER SOILSTICE QUESTIONNAIRE



(Please complete and return with the soil samples in the freepost envelope.)

Name: _____

Date collected: _____

LOCATION (home or workplace garden)

Address: _____

_____ Postcode: _____

NB: If you have a compost heap or use compost in **your own garden** please sample from these.

SAMPLE 1

☐ pot/planter ☐ border ☐ bag of compost ☐ bag of manure ☐ compost heap

Sample details (e.g. plant or bulb type in pot, brand of compost or manure, composted vegetation)

SAMPLE 2

☐ pot/planter ☐ border ☐ bag of compost ☐ bag of manure ☐ compost heap

Sample details (e.g. plant or bulb type in pot, brand of compost or manure, composted vegetation)

Thank you for taking part in this Summer Solstice soil sampling project! If you would **not** like to be emailed project updates and a link to the online interactive map to view your anonymized data points, please mark a cross in this box: ☐

Appendix 10: Google form sent out four days after the first air sampling round asking participants for feedback on their sampling experience.

Questions Responses 119



Science Solstice feedback

Thank you again for taking part in Science Solstice and contributing to an important and fascinating part of my PhD! As a scientist it is very important to evaluate my work- to understand what went well and what went badly, and to learn how to improve in future. I would very much appreciate it if you could take 1 minute to give me your feedback on your Science Solstice experience!

If you DID NOT send back your air sampler(s) could you tell me the reason?

- ☐ Air sampling pack did not arrive in time/at all
- ☐ Forgot/was too busy/was on holiday on the day
- ☐ Label on top of label caused confusion on how to uncover air sampler(s)
- ☐ Air sampler(s) blew away
- ☐ No freepost return envelope in the sampling pack
- ☐ It was too windy/it was raining
- ☐ Other...

Were the email reminders helpful?

- ☐ Yes
- ☐ No
- ☐ Did not receive any

Was the text reminder the evening before useful?

- ☐ Yes
- ☐ No
- ☐ Did not receive it

If I repeated Science Solstice would you like to part? If yes, please enter your email address below:

Short answer text

Any other comments- good or bad!

Long answer text

Appendix 11: Participant feedback on the first air sampling round provided in the additional comments box of the Google form sent out on 25th June 2018.

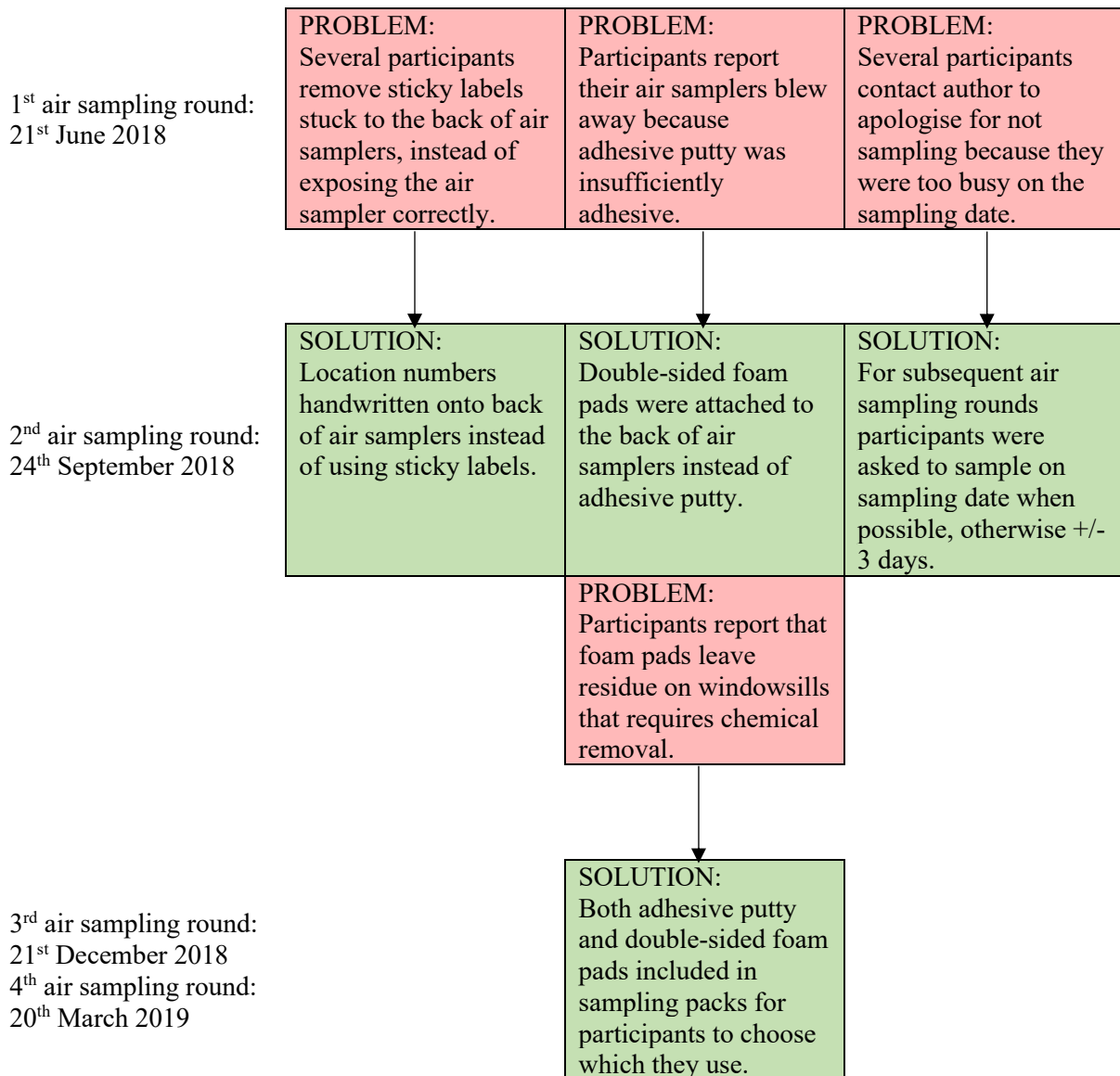
1. More samplers as a contingency if/when we had problems with the first two
2. A practice experiment with some people to find out what could go wrong
After removing white covers I paper-clipped these face-to-face so as to keep uncontaminated until the evening when they were replaced on the sticky sheets. I do not know where other participants kept theirs (e.g. face-up or face-down / on windowsill or in a drawer) and it might not make much difference as long as they were inside the house (most likely, or they would have blown away!) but it might be something to consider?
All went OK. Suggest using thicker plastic as the hot weather tended to curl up the corners making it easier to blow away. It would be nice to have a dot map showing where the air samplers were located by volunteers etc. No other issues - you will sort out the 'label' problem so fingers crossed that you get the results you want. PS worked on <i>Aspergillus fumigatus</i> isolated from self-heating coalspoil sites 50 years ago so am interested in what you find anyway.
As spare sampler in the pack to replace any that went bad. Double sided tape as well as blue tac
Best wishes with your PhD project! I think it's excellent to use the general public to facilitate data collection. Well done!
blue tack didn't stick to UPVC very well
Difficult to leave the sticker unattended especially at the public area - Risk to be removed by cleaners etc
Enjoy the sampling.
Enjoyed taking part, thanks. I managed to get a finger print on one of my samples (sorry) - actually realised (too late) that there was a non sticky part of the backing paper from which we were presumably supposed to peel, but as this wasn't marked, I didn't realise this until after I'd put out the samples. Could this be marked somehow to make the unpeeling easier? Also, wondering if these same samples could be used for other studies, e.g. looking at pollen counts/pollen species to develop/refine pollen maps for epidemiology research? Anyway, good luck with the analysis, I'll look forward to hearing more in due course!
Everything was well explained.
Exciting project! We look forward to hearing about what you find!
Firstly, this is a great project, and I hope you get some nice results from it. I don't know how feasible this is, but you might want to consider adding an edge around the sampler to hold on to, so people don't have to be concerned about the fingerprint and contamination issue. From an ecological perspective, I suggest to include replicates for each location.
From what I could gather from your e-mails, I guess you will end up with "clumped data", i.e. mostly around Oxford, maybe some other UK science hotspots ;-), and then Leipzig and Ontario? It would be really interesting to see how you deal with this in the interpretation/analysis of the data. And congrats on choosing a fascinating project, and a great citizen science approach to boot!
Fun to take part!
Fun! Next time I'll be back in Kent.
Good! Also I think I didn't sign up for updates. Please add me :)
Great project - was a tiny bit confused by the white backing at first, but your instruction sheet helped with that. Otherwise, the issue of blutack and making it stick to surfaces as I didn't want to touch the sticky top. Otherwise, very well communicated and explained what I needed to do.
Great project! Good luck. Hope our samples are useful (the cat stood on one of them!). Happy to help with anything in the future.
Great to be involved. We should be doing more of citizen science in general!

Great to take part.. Feel that I contaminated my sample through a level of ignorance over removal of label etc.. Next time make it ultra idiot proof.. You did to an extent but silly people on the school run may have struggled.. Sorry!
Happy to help. Good luck.
Home air sampler left out much longer than work air sampler - held up at work so air sampler at home was left out for just over the maximum time. I hope this won't affect / influence anything too much. I thought the instructions were really clear, you provided a great amount of background information and made volunteers feel a part of the project.
I agree it was difficult not to get fingerprints on it
I also struggled with the fingerprint thing. Possibly having a marked edge that it was okay to touch could have helped with this? Also I emailed about not having downstairs windowsills to which you replied promptly and so I used other objects, this may have been outlined somewhere in the information but I didn't (and still haven't) read it properly. Just looked at the basic instructions...
I enjoyed taking part and look forward to seeing the results.
I had no problems removing the backing or with the sampler blowing away - even though it was a windy day.
I look forward to seeing your results. Could you let me know if my samples arrived with you please. Be great to see pics of what my samples look like on the plates. Would you like me to send you pics of the two sites to add to your photo collection. (Will also send this in an email in case you don't look at feedback for a while.)
I really like this experience and will definitively be happy to help in the future if needed!
I think I would maybe add a replacement sampling thingy, since then if the first one is screwed up, or one blew away, you could still have 2 samples from each place...
I think this was really well organised and you've done a brilliant job. But one thing I have learnt over the years, is to never underestimate people's creativity in making mistakes or misunderstanding! You can be crystal clear and someone will always muck it up so don't take it personally. All the best with the PHD.
I think you have been very diligent but I also think you have not captured as much associated data as you could have e.g. start and end time of sample while unlikely to have influenced results may have been useful as I expect some people did not stick to the 6-8 hours (I left home 8 am and did not return until 10 pm) , local weather (I would have provided tick box of possibilities with a free text box comments), proximity to factors which might effect results e.g. arable agriculture fields with high probability of chemical sprays or what ever. Good luck with the PhD
I think you've had enough feedback on the tricky bits already. My samplers had a non-sticky edge (not sure if they all do), and it was easiest to peel from that edge.
I thought comms were good, and excellent excitement generated on Twitter! I wonder if numbered steps *on* the collector might have helped avoid fingerprints (like mine!) - I.e. (printed on film). "1. Stick this side down to windowsill" or similar. (on white side) "2. peel this bit off and retain". As you would see on phone screen protectors. I know it shouldn't be hard but I managed to do it the wrong way round, and I spent years in a micro lab...! Well done for thinking up such an interesting experiment -looking forward to the results!
I thought it worked really well but the blu-tack is not suited for dusty or wet surfaces outside so I'd strongly suggest double-side foam tape cut into square (it has a peelable backing) it's what I used and it stayed just fine. I also found having a little note on the underside facing up saying 'Air-sampling please don't touch' helped.
I thought the instructions were very clear - even the white label! Was wondering why two samples and whether you needed these to be far apart or whether two very close together (e.g. as replicates) would be useful? Or having a control (inside) to compare with?
I thought the label thing was clear and obvious (particularly with the photos) but my friend, who did sample 2 for me in his flat, somehow found it very confusing. Noting to not do it overnight would have been helpful. The two sample thing may have put some people off as some people

might not have two places to do it - I could not do one at work as I work in an upper floor office. I really enjoyed taking part though as a non-scientist - a simple task and I'm really interested in the results. I liked the photo instructions and the info sheet. The poster didn't seem that worthwhile though because by the time we received it it would have been a bit late for other people to take part.
I was happy to be a part of it and am looking forward to the results!
I was worried about 'effort' because I was able to put my sampler at home out very early in the morning and then got home super late, whereas at work I had meetings in the morning that meant it didn't get put out till 11 and taken in as I caught train home, so the time taken to collect the particles was mismatched with my work sample. For surveys I know 'effort' matters and it might be similar here. Can we have a bit to fill in how much time they were out or does that not matter? Otherwise, super easy to do and think it's a great idea!
It was clearly explained and easy to do, except for the sticky labels, which were difficult to peel off.
It was tricky to remove the white backing without touching the sticky surface - I think including more Blu-tack would solve that.
it's hard to set air samples for 8 hours in two different locations; even if it's home and work it is unusual to have the exact time for both; perhaps more info on the allowance for time (like between 6 and 10?) would have helped
Just unfortunate that both samplers blew away :(
Look forward to seeing results!
Also, might be good to tell people to wipe the window ledge before sticking the thing. One of mine was so covered in pollen that it made the sticky surface immediately non sticky... the window sill needs to be pretty clean and dry.
Good luck!
more Blu-Tack :)
My outside windowsills are made of rough stone so blue tack doesn't work. A bit more information about why windowsills were suggested would have helped me choose an alternative spot. South Uist is Very Windy!
I struggled a bit to get the sticky surface completely re-covered after the sampling. Making the backing bigger than the sticky sampler might help.
Having said all that, it was easy enough and I would do it again.
Nice citizen science project!
nice work, well done; perhaps indeed try to find a solution for the blu Tack, not sticky enough (for eg stone)
no confusion about what label to remove, you've explained very well!
No issues. Was a doddle.
Nothing particular apart from that I felt the reminder text came in a bit late in the day, as I received the letter at my workplace, and forgot to bring it home the day before, so I had to return home on the Summer Solstice to deploy it. I think an e-mail/text reminder at noon on the day before and a text reminder in the evening is better. Good luck with your research!
Noticed about the finger prints and tried to avoid it by pressing down the Blu tack with the plastic backing which seemed to work. Was fun, would like to see results in any way possible
Only comment would be something that you have already identified in your email, that it was quite difficult to avoid getting finger prints on the clear sticky screen
Perhaps next time make the peely away thing a different colour from the label so people aren't confused. Enjoyed doing the experiment and made me feel very important!
Please keep me on your email list for updates on project results.
Really enjoyed being part of it
Really enjoyed being part of your survey - looking forward to hearing about the findings. Got my granddaughter involved to provide the second site in town as I'm retired! Best wishes, Mary

Really enjoyed taking part - and reading the science bit on the information sheet. Great for my son to learn about too
Really fun and easy project, and your enthusiasm throughout was great!
Really nice and interesting experiments, easy to participate even far from your lab! I had just a question on the fact that it was impossible for me to not touch the sampler with my fingers. May it bias the results!?
Would be happy to follow your results. Good luck.
Really well organised and sounds a great project - good luck with the analysis!
so wonderful to be a part of a fascinating project
sorry I think we contaminated the sample with fingerprints, I must have missed that in the instructions, other than that it was very well organised and simple to take part
The instructions were very clear and easy to understand. Really satisfying to be involved in some research! Best of luck with your studies and I look forward to seeing the results.
The three points in your email (confusion over labels etc) were all my experience too!
There is a large community of home educating families who love citizen science and use it for study. If you're looking for even more people let me know and I can help to publicise it within our community groups. Additional info with experiments/sampling kits is great to help the children to learn. Good luck with your studies and career
Unfortunately I was one of the klutzes who peeled off the white address label rather than the whole top cover - pretty stupid, but instructions need to be foolproof, and it needs to be remembered that most of the people involved know nothing (as in NOTHING) about air sampling and hence aren't well equipped to know they have done something daft. Hope you got good coverage regardless - and get useful results.
Very impressed by your commitment to keeping people informed.
Very interesting project
Very interesting project and delighted to take part. As you are already aware, there was an issue about which label to peel off, though to be fair to you, instructions were provided in the pack; for next time, better to make it idiot-proof! All good wishes for your research.
Very interesting project. Great fun
We also struggled not to get finger prints on the sticky side but other than that everything was fine and the instructions were clear.
We love to get involved with physical hands on activities - so much more fun than simply filling in a survey. Well done and all the best with your research.
Well done, envelope with two samples on its way.
Were you still able to use the experiment even though some people took the wrong label off at first, but exposed the correct label after getting your later email?
What fun!
Will be interested to hear the results
Would be interested in finding out your results
Would definitely be interested in seeing the results (fellow scientist here...)
Yeah I think you mentioned before, but I got confused which location was which sample for a second, but luckily I'd filled out the info sheet before I stuck them out so I knew which was which. But if I hadn't very likely I would have forgotten.

Appendix 12: Timeline of changes made to air sampling methodology across the four air sampling rounds as a result of participant feedback.



Appendix 13: Maximum air temperatures for dates that air samples were collected, according to Met Office daily climate data for sample location.

Sampling round and dates samples were collected	Number of samples	Mean maximum air temperature (range; °C)
Summer	710	18.6 (14.0-27.2)
20 th June 2018	2	15.2 (14.5-15.6)
21 st June 2018	676	18.5 (14.0-21.4)
22 nd June 2018	26	21.1 (16.2-21.9)
23 rd June 2018	5	21.5 (20.4-22.3)
28 th June 2018	1	26.3
1 st July 2018	1	27.2
Autumn	398	16.0 (12.5-23.7)
17 th September 2018	2	20.1 (20.1-20.1)
20 th September 2018	4	20.2 (20.0-20.4)
22 nd September 2018	1	13.5
23 rd September 2018	4	13.2 (12.7-13.7)
24 th September 2018	258	15.2 (12.6-16.6)
25 th September 2018	55	16.7 (13.8-18.1)
26 th September 2018	33	20.7 (18.0-23.0)
27 th September 2018	16	19.9 (13.0-23.7)
28 th September 2018	14	15.3 (12.5-17.6)
29 th September 2018	3	15.2 (15.0-15.4)
30 th September 2018	6	14.4 (13.7-14.8)
1 st October 2018	2	13.8 (13.8-13.8)
Winter	321	10.0 (2.7-13.5)
12 th December 2018	2	7.7 (7.7-7.7)
14 th December 2018	3	5.2 (3.9-7.4)
16 th December 2018	2	7.4 (7.4-7.5)
17 th December 2018	159	10.5 (7.8-13.0)
18 th December 2018	15	9.8 (9.1-10.3)
19 th December 2018	52	8.5 (6.0-10.3)
20 th December 2018	40	10.8 (2.8-13.0)
21 st December 2018	12	10.0 (7.9-11.9)
22 nd December 2018	19	10.2 (7.6-12.1)
23 rd December 2018	2	13.5 (13.5-13.5)
24 th December 2018	3	8.2 (4.5-10.1)
25 th December 2018	2	10.2 (10.2-10.2)
28 th December 2018	3	9.7 (9.6-9.8)
6 th January 2019	4	10.2 (9.8-10.4)
17 th January 2019	1	5.1
19 th January 2019	2	2.8 (2.7-2.8)
Spring	461	13.3 (8.5-18.8)
13 th March 2019	2	12.1 (12.0-12.1)
17 th March 2019	2	10.1 (10.1-10.1)
18 th March 2019	4	10.2 (8.8-11.3)
19 th March 2019	21	12.3 (10.0-13.5)
20 th March 2019	288	13.8 (9.3-18.8)
21 st March 2019	76	13.3 (11.3-16.2)
22 nd March 2019	44	11.0 (8.5-14.1)
23 rd March 2019	11	11.9 (9.4-13.8)
24 th March 2019	3	11.7 (11.3-12.4)
25 th March 2019	2	11.9 (11.9-11.9)
26 th March 2019	2	14.0 (13.9-14.1)
27 th March 2019	2	12.5 (10.1-14.9)
28 th March 2019	2	15.3 (15.3-15.3)
24 th April 2019	2	16.6 (16.6-16.6)

Appendix 14: Details of 736 *Aspergillus fumigatus* isolates cultured from soil samples that grew at tebuconazole 6 mg/L; including the sample they were grown from, the county of the sampling location, the garden location from which the sample was collected and the *cyp51A* polymorphisms that were detected.

Sample	County, north to south	Garden location sampled from	<i>cyp51A</i> polymorphisms	Number of ARAf isolates
077-01	Aberdeenshire	compost heap	TR ₃₄ /L98H Failed to re-grow	12 1
077-02	Aberdeenshire	border	TR ₃₄ /L98H	1
057-02	Perth and Kinross	border	TR ₃₄ /L98H TR ₅₃	4 1
003-01	Clackmannanshire	compost heap	TR ₃₄ /L98H	2
173-01	Stirlingshire	compost heap	TR ₃₄ /L98H	55
086-02	Midlothian	pot/planter	TR ₃₄ /L98H Failed to re-grow	2 1
040-02	Glasgow	border	TR ₃₄ /L98H WT Failed to re-grow	1 2 1
214-01	South Lanarkshire	pot/planter	TR ₃₄ /L98H	2
214-02	South Lanarkshire	compost bag	TR ₃₄ /L98H	4
226-01	Belfast	border	Failed to re-grow	2
226-02	Belfast	border	Failed to re-grow	4
244-02	Lancashire	pot/planter	TR ₃₄ /L98H	3
045-01	Lancashire	pot/planter	TR ₃₄ /L98H I242V WT Failed to re-grow	3 2 2 1
045-02	Lancashire	pot/planter	TR ₃₄ /L98H TR ₃₄ I242V C270R WT Failed to re-grow	5 1 3 1 13 2
068-02	Lancashire	pot/planter + compost bag	TR ₃₄ /L98H	1
137-02	North Yorkshire	border	TR ₃₄ /L98H	1
165-01	Lincolnshire	compost heap	TR ₃₄ /L98H TR ₄₆ /Y121F/T289A (TR ₄₆)2/Y121F/M172I/T289A/G448S Failed to re-grow	1 3 1 3
126-01	West Yorkshire	compost bag	TR ₃₄ /L98H	1
126-02	West Yorkshire	compost heap	TR ₃₄ /L98H	7
048-02	Greater Manchester	pot/planter	Failed to re-grow	6

024-02	Greater Manchester	pot/planter	TR ₃₄ /L98H	3
182-02	Greater Manchester	pot/planter + compost bag	TR ₃₄ /L98H/D481N TR ₃₄ /L98H Failed to re-grow	1 21 6
007-02	Merseyside	border	TR ₃₄ /L98H Failed to re-grow	2 1
062-02	Greater Manchester	pot/planter	TR ₃₄ /L98H	2
015-02	Greater Manchester	pot/planter	TR ₃₄ /L98H/K240R TR ₃₄ /L98H/A460S TR ₃₄ /L98H Failed to re-grow	1 1 8 1
015-03	Greater Manchester	border	TR ₃₄ /L98H/K240R TR ₃₄ /L98H	1 29
015-05	Greater Manchester	border	Failed to re-grow	1
015-16	Greater Manchester	pot/planter	TR ₃₄ /L98H	4
198-02	South Yorkshire	compost heap	TR ₃₄ /L98H	1
129-01	South Yorkshire	compost bag	TR ₃₄ /L98H Failed to re-grow	2 2
106-01	Cheshire	pot/planter	TR ₃₄ /L98H	2
102-01	Derbyshire	pot/planter	Failed to re-grow	1
023-02	Cheshire	compost heap	TR ₃₄ /L98H	17
167-02	Cheshire	compost bag	TR ₃₄ /L98H	2
074-02	Lincolnshire	border	TR ₃₄ /L98H (TR ₃₄) ² /L98H Failed to re-grow	11 1 18
078-01	Nottinghamshire	pot/planter	TR ₃₄ /L98H WT Failed to re-grow	4 5 5
216-01	Lincolnshire	pot/planter	TR ₃₄ /L98H	1
231-01	Nottinghamshire	compost heap	TR ₃₄ /L98H	1
160-01	Norfolk	compost heap	TR ₃₄ /L98H Failed to re-grow	26 4
160-02	Norfolk	compost bag	TR ₃₄ /L98H	9
255-01	Shropshire	compost heap	TR ₃₄ /L98H/K372R TR ₃₄ /L98H TR ₄₆ /Y121F/T289A not Afum?	1 12 1 1
210-01	Norfolk	pot/planter + compost bag	TR ₃₄ /L98H	2
207-01	West Midlands	compost bag	TR ₃₄ /L98H/D481N TR ₃₄ /L98H Failed to re-grow	1 5 6
018-02	West Midlands	border	TR ₃₄ /L98H	2

101-01	Worcestershire	border	TR ₃₄ /L98H	10
082-02	Cambridgeshire	compost heap	TR ₄₆ /Y121F/T289A	1
032-01	Cambridgeshire	pot/planter	TR ₃₄ /L98H/P443L	1
			TR ₃₄ /L98H	2
			WT	2
054-02	Essex	border	TR ₃₄ /L98H	3
			WT	1
			Failed to re-grow	1
135-01	Gloucestershire	pot/planter	TR ₃₄ /L98H	6
			Failed to re-grow	2
135-02	Gloucestershire	pot/planter	TR ₃₄ /L98H	1
			Failed to re-grow	2
097-01	Gloucestershire	pot/planter + compost bag	TR ₃₄ /L98H	6
149-01	Hertfordshire	pot/planter + compost bag	TR ₃₄ /L98H	2
			TR ₃₄	1
			TR ₄₆ /Y121F/T289A	1
			Failed to re-grow	5
149-02	Hertfordshire	pot/planter	TR ₃₄ /L98H	1
055-02	Oxfordshire	compost heap	TR ₃₄ /L98H/T289A/I364V/G448S	1
			TR ₃₄ /L98H	28
			TR ₄₆ /Y121F/T289A	1
038-01	Oxfordshire	compost bag	TR ₃₄ /L98H/P394R	1
			TR ₃₄ /L98H/K421R	1
			TR ₃₄ /L98H	28
150-01	Pembrokeshire	border	TR ₃₄ /L98H	4
			Failed to re-grow	1
150-02	Pembrokeshire	compost heap	Failed to re-grow	1
158-02	Buckinghamshire	compost heap	TR ₃₄ /L98H/T289A/I364V/G448S	5
			TR ₃₄ /L98H	7
			TR ₄₆ /Y121F/T289A	3
			TR53	1
			not Afum?	1
			Failed to re-grow	2
146-02	Buckinghamshire	compost bag	Failed to re-grow	3
072-01	Oxfordshire	compost heap	TR ₃₄ /L98H	1
033-02	Mid Glamorgan	compost bag	TR ₃₄ /L98H	8
071-02	Essex	compost bag	TR ₃₄ /L98H	2
009-01	Oxfordshire	compost heap	TR ₃₄ /L98H/N406M	1
			TR ₃₄ /L98H	3
			WT	1
			Failed to re-grow	3
009-02	Oxfordshire	pot/planter	TR ₃₄ /L98H	1
			Failed to re-grow	1
029-01	Oxfordshire	pot/planter	TR ₃₄ /L98H	2
029-02	Oxfordshire	compost bag	TR ₃₄ /L98H/F404V	1

			TR ₃₄ /L98H	5
151-01	Greater London	pot/planter + compost bag	Failed to re-grow	3
229-01	Berkshire	compost heap	TR ₄₆ /Y121F/T289A	1
084-02	Oxfordshire	pot/planter	TR ₃₄ /L98H	1
			WT	1
123-02	Greater London	pot/planter	Failed to re-grow	5
134-01	South Gloucestershire	compost bag	TR ₃₄ /L98H	1
253-01	Surrey	border	TR ₃₄ /L98H	1
099-01	Bristol	pot/planter	TR ₃₄ /L98H	4
249-01	Berkshire	compost heap	TR ₃₄ /L98H	2
161-01	Kent	compost heap	TR ₃₄ /L98H	1
130-01	Greater London	border	Failed to re-grow	1
010-01	Greater London	pot/planter	TR ₃₄ /L98H/P443L	1
			TR ₃₄ /L98H	6
			Failed to re-grow	2
010-02	Greater London	compost bag	TR ₃₄ /L98H/F404C/F459S/A460S	1
			TR ₃₄ /L98H/N406D	1
			TR ₃₄ /L98H	3
087-01	Greater London	border	not Afum?	3
087-02	Greater London	compost heap	TR ₃₄	1
			TR ₃₄ /L98H	8
			TR ₄₆ /Y121F/T289A/S363P/I364V/G448S	1
			TR ₄₆ /Y121F/T289A	3
			TR ₁₃₀ /D430G	4
257-02	Berkshire	compost bag	TR ₃₄ /L98H	3
			TR ₄₆ /Y121F/T289A	1
056-02	Greater London	compost bag	TR ₃₄ /L98H	30
195-02	Wiltshire	border	TR ₃₄ /L98H	12
039-01	Somerset	compost heap	TR ₃₄ /L98H	3
127-01	Greater London	pot/planter + compost bag	TR ₃₄ /L98H	1
127-02	Greater London	compost heap	TR ₃₄ /L98H	1
			TR ₄₆ /Y121F/T289A	1
053-01	Surrey	compost heap	TR ₃₄ /L98H	3
012-02	Surrey	compost bag	TR ₃₄ /L98H	4
052-01	Kent	border	TR ₃₄ /L98H	1
052-02	Kent	pot/planter	not Afum?	2
114-02	Devon	compost bag	TR ₃₄ /L98H/Q191E	1
			TR ₃₄ /L98H	3
136-02	Somerset	compost bag	TR ₃₄ /L98H	2
			Failed to re-grow	1
110-02	Devon	compost heap	TR ₃₄ /L98H	3
			Failed to re-grow	1
256-01	Devon		TR ₃₄ /L98H/R196L	1

		border/compost bag	TR ₃₄ /L98H	7
238-02	Hampshire	pot/planter	TR ₃₄ /L98H	1
073-01	Sussex	compost heap	TR ₄₆ /Y121F/M178W/T289A/S363P/I364V/G448S TR ₄₆ /Y121F/T289A/S363P/I364V/G448S	1 3
091-02	Sussex	pot/planter + compost bag	TR ₃₄ /L98H	1
199-01	Hampshire	compost heap	TR ₃₄ /L98H	30
107-02	Dorset	compost bag	TR ₃₄ /L98H	2
042-01	Isle of Wight	compost bag	TR ₃₄ /L98H	3
176-01	Cornwall	compost heap	TR ₃₄ /L98H	13
124-01	Devon	pot/planter	TR ₃₄ /L98H	1



Appendix 16: Certification for participation in Young Entrepreneur Scheme (YES) 2020 with business proposal “TerraGrow” – mycorrhizal mixes of fungi for promoting growth of newly-planted trees.



This is to certify that
Jennifer Shelton
participated in the YES 2020 Competitions

A handwritten signature in blue ink, appearing to read "S Mosey".

Professor Simon Mosey
on behalf of the Organising Group



Appendix 17: Co-authored papers published during PhD that are relevant to this thesis.

[Pre-print] **Shelton, J. M. G.**, Collins, R., Uzzell, C. B., Alghamdi, A. & Dyer, P. S. (2021): Citizen-science surveillance of triazole-resistant *Aspergillus fumigatus* in UK residential garden soils. *bioRxiv* 1–21.

Ghosh, P., Brookes, L., Edwards, H., Fisher, M., Jervis, P., Kappel, D., Sewell, T., **Shelton, J. M. G.**, Skelly, E., and Rhodes, J. (2020): Cross-disciplinary genomics approaches to studying emerging fungal infections. *Life* **10**(12), 1–16.

Brackin, A., **Shelton, J. M. G.**, Abdolrasouli, A., Fisher, M., and Sewell, T. (2020): A Low-Cost Tebuconazole-Based Screening Test for Azole-Resistant *Aspergillus fumigatus*. *Current Protocols in Microbiology* **58**(1), 1–12.

Shelton, J. M. G., Fisher, M., Singer, A. (2020): Campaign-Based Citizen Science for Environmental Mycology: the ‘Science Solstice’ and ‘Summer Soil-stice’ Projects to Assess Drug Resistance in Air and Soilborne *Aspergillus fumigatus*. *Citizen Science: Theory and Practice* **5**(1), 20.

Sewell, T., Zhang, Y., Brackin, A., **Shelton, J. M. G.**, Rhodes, J., Fisher, M. (2019): Elevated prevalence of azole resistant *Aspergillus fumigatus* in urban versus rural environments in the United Kingdom. *Antimicrobial Agents and Chemotherapy* **63**, 1-3.

Nash, A., Sewell, T., Farrer, R., Abdolrasouli, A., **Shelton, J. M. G.**, Fisher, M., Rhodes, J. (2018) MARDy: Mycology Antifungal Resistance Database. *Bioinformatics* **34**(18), 3233-3234.

Appendix 18: Blogs and articles published during PhD that are relevant to this thesis.

Imperial Medicine Blog (2021) – “The future is fungal”

<https://blogs.imperial.ac.uk/imperial-medicine/2021/04/28/the-future-is-fungal/>

British Society of Antimicrobial Chemotherapy (2020) – “The forgotten superbug”

bsac.org.uk/the-forgotten-superbug-is-it-time-to-bring-fungal-infections-out-of-the-dark

Imperial Medicine Blog (2021) – “Covid-19 and fungal lung infections”

<https://blogs.imperial.ac.uk/imperial-medicine/2020/05/27/covid-19-and-fungal-lung-infections-a-case-study-on-aspergillosis/>

Society for Applied Microbiology magazine (2019) – “Science Solstice: sticky side up”

issuu.com/societyforappliedmicrobiology/docs/sfam_microbiologist_march_2019_web

Imperial Medicine Blog (2021) – “Strength in numbers”

<https://blogs.imperial.ac.uk/imperial-medicine/2019/01/09/strength-in-numbers-monitoring-of-fungicide-resistance-with-the-help-of-500-citizen-scientists/>