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SCIENTIFIC REPORT

Impact of the use of azole fungicides, other than as human medicines, on the development of azole-resistant *Aspergillus* spp.

European Food Safety Authority (EFSA) | European Centre for Disease Prevention and Control (ECDC) | European Chemicals Agency (ECHA) | European Environment Agency (EEA) | European Medicines Agency (EMA) | European Commission's Joint Research Centre (JRC)*

Correspondence:
EFSA: pesticides.peerreview@efsa.europa.eu

ECDC: press@ecdc.europa.eu

ECHA: press@echa.europa.eu

EEA: https://www.eea.europa.eu/en/about/contact-us

EMA: https://www.ema.europa.eu/en/about-us/contacts-european-medicines-agency/send-question-european-medicines-agency

JRC: jrc-press@ec.europa.eu

*JRC provided scientific support to the Agencies.

The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

The use of azoles in the European Union and European Economic Area (EU/EEA) other than as human medicines has raised concerns about emergence and spread of azole-resistant *Aspergillus* species. EU agencies, with the support of JRC, reviewed the evidence and provided conclusions and recommendations on this topic. Although incomplete, data from 2010 to 2021 showed that around 120,000 tonnes of azoles were sold in EU/EEA for uses other than as human medicines. The majority are used as plant protection products (119,000 tonnes), with a stable temporal trend. Evidence supported a link between environmental azole exposure and cross-resistance selection to medical azoles in *Aspergillus* species (primarily shown for *A. fumigatus*). Prevalence of azole-resistant *A. fumigatus* in human *A. fumigatus* infections ranges from 0.7% to 63.6% among different disease presentations and geographic regions; mortality rates range from 36% to 100% for invasive aspergillosis (IA). It was concluded that azole usage outside the human domain is likely or very likely to contribute to selection of azole-resistant *A. fumigatus* isolates that could cause severe disease like IA. Environmental hotspots for resistance selection were identified, including stockpiling of agricultural waste and their possible use as soil amendment/fertiliser for certain agricultural crops (for plant protection products) and freshly cut wood (for biocides). Recommendations were formulated on measures to prevent and control selection of azole resistance in *A. fumigatus*, including implementation of good agricultural/horticultural practices, proper agricultural and wood waste storage and management, and on approval of new azole fungicides or renewal of existing fungicides. Recommendations on topics to be covered by studies provided when submitting applications for the approval of azole fungicides were listed. For the evaluation of such studies within the approval procedure, a preliminary framework for risk assessment was developed and should be further refined. Data gaps and uncertainties were identified, alongside with respective recommendations to address them.

KEYWORDS

antifungal, *Aspergillus*, azole resistance, biocide, fungicide, hotspot, industrial chemical, invasive aspergillosis, plant protection product, veterinary medicinal product

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SUMMARY

Aspergillus spp., a filamentous fungus widespread in the environment, is one of the causative agents of the most serious fungal infections in humans, aspergillosis, which occurs primarily in people with compromised immune systems or lung diseases. *A. fumigatus* is the most widely represented species and is the primary cause of aspergillosis in the EU, including invasive aspergillosis (IA), chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA). Azoles represent the primary treatment options for aspergillosis. Other than their use as human medicines, azoles are widely used as fungicides within plant protection products (PPPs), for the control of fungal diseases in plants, as biocides within biocidal products (BPs), in particular as wood preservatives, as veterinary medicinal products (VMPs), to treat fungal diseases in companion and food-producing animals, and as industrial chemicals (e.g. as intermediates, dyes or in cosmetics).

The emergence of azole-resistant *Aspergillus* spp. is diminishing the effectiveness of azole therapy of patients with aspergillosis. Azole resistance in *Aspergillus* spp. may develop in the host during azole therapy (patient route) but may also develop in the environment following exposure to azole fungicides (environmental route). For this reason, the European Commission requested the European Food Safety Authority (EFSA), the European Chemical Agency (ECHA), the European Medicines Agency (EMA), the European Centre for Disease Prevention and Control (ECDC) and the European Environment Agency (EEA), with the scientific support from the European Commission's Joint Research Centre (JRC), to collaborate and produce this scientific report. The EU agencies, with the support of JRC, reviewed the evidence and provided conclusions and recommendations on this topic.

The main chemical classes of azoles studied in this report and deemed relevant/responsible for selection of resistance in *Aspergillus* spp., are triazoles and imidazoles. Data collected from competent authorities of EU/EEA Member States, although incomplete, showed that, overall, around 120,000 tonnes of azoles were reported to be sold on the EU/EEA market between 2010 and 2021 for uses other than as human medicines. Most of the azoles are used as PPPs (more than 119,000 tonnes), with a stable trend, on average 10,000 tonnes a year, with alterations in the different countries. It was not possible to draw any firm conclusions regarding geographical trends in sales and use of azoles as PPP, BP, VMP and industrial chemicals, as most of the substances were used throughout the EU/EEA. A rough comparison made for year 2021 showed that the amount of triazole and tetrazole derivatives for systemic use consumed by humans (including both the community and hospital sectors) was about 1000 times smaller than the amount of azole substances reported for the other above-mentioned uses.

Evidence on the mechanisms of resistance to azole fungicides present in *Aspergillus* spp. isolates from the human, agricultural, environmental and animal domains was reviewed. The most frequently identified molecular resistance mechanism in azole-resistant *A. fumigatus* (ARAf) isolates from azole-naïve patients was the TR_{34}/L98H mutation, followed by the TR_{46}/Y121F/T289A mutation, as well as the G54E and Y121F mutations.

There is substantial evidence that supports a link between azole resistance selection through azole fungicide exposure in the environment and cross-resistance selection to medical azoles in *Aspergillus* species (environmental route of resistance selection). This link has been primarily shown for *A. fumigatus* and remains less clear for other *Aspergillus* species. It has been primarily shown for PPPs, while it is less clear for BPs and industrial chemicals and seems unlikely for VMPs.

Information on the prevalence of ARAf in human *A. fumigatus* infections was also reviewed, which varied among the different presentations of the disease and between geographic regions: 0.7%–63.6% for IA, 5.9%–59.2% for CPA and 2.3%–42.8% for ABPA. This variation can be attributed to various factors such as differences in antifungal exposure, environmental factors and the emergence of specific azole-resistant strains, but also due to detection bias in certain areas. Risk factors associated with aspergillosis due to ARAf, and more generally azole-resistant *Aspergillus* spp., include underlying immunosuppression and certain disease conditions. Exposure to agricultural sites, woodwork and ground maintenance can potentially increase the risk for the acquisition of azole-resistant *Aspergillus* spp. Clinical implications of azole resistance have been reported in IA (36%–100% mortality rates) but are less well documented in CPA and ABPA.

Overall, available evidence supports the hypothesis that transmission of ARAf occurs from the environment to humans. It was concluded that azole usage outside the human domain is likely or very likely to contribute to the selection of ARAf isolates that could cause severe disease such as IA, but the extent of this contribution needs to be better understood.

Concentrations of selected azoles in European surface and groundwaters identified exceedance of aquatic safety thresholds in some surface waters, with fewer exceedances of the quality threshold in groundwater.

Ecological selection dynamics for ARAf include activity of azole fungicides against *A. fumigatus*, substrates that supports the growth of *A. fumigatus,* and the presence of fungicide residues that exceed the predicted no effect concentration for resistance selection ($PNEC_{res}$). These dynamics support azole resistance selection in specific scenarios, referred to as environmental hotspot for resistance selection, i.e. environments that support the selection and dispersal of azole-resistant *A. fumigatus*. For PPPs, the relevant scenarios identified include stockpiling of agricultural waste and their possible use as soil amendment or fertiliser for various agricultural crops such as indoor growing fruiting vegetables, wine grape, maize, sugar beet, olives, pome fruit, citrus fruit and field heaps. For BPs, the relevant scenario identified include freshly cut wood. For VMPs and industrial chemicals, no relevant scenarios were identified.

A number of recommendations were formulated on measures to prevent and control the selection of azole resistance in *A. fumigatus* in the environment.

Some recommendations relate to the use and approval of azoles, in particular considering carefully the need to use an azole substance in a PPP or BP, providing or performing a prior assessment of the risks for cross-resistance with antifungals used in human medicine before approving a new fungicide or renewing an existing approval, and considering including

specific requirements related to the public health risks of antifungal resistance within regulatory requirements related to approval of new fungicides or renewal of existing approvals. It was also recommended to support research and development of both new fungicides with novel mechanism of action that do not have cross-resistance with antifungals used in human medicine, and new antifungal medicinal products active against azole-resistant *Aspergillus* spp.

For the agricultural sector, several measures were recommended, such as implementing and further developing Good Agricultural/Horticultural Practices in professional agriculture and horticulture, ensuring controlled storage of organic waste, proper waste management and responsible use and disposal of azole-treated products. Regarding the application of azoles as biocides in the wood sector, recommendations were formulated regarding application conditions and implementation of proper wood waste management.

This report also lists topics to be covered by the studies or information that could be provided by applicants when submitting applications for the approval of azole substances for uses other than as human medicines. Among them, performance of in vitro susceptibility testing based on validated and standardised methods can already be recommended to flag active substances that may potentially contribute to cross resistance. It was recommended that further specific guidance be developed to provide technical specifications for specific studies to be submitted within approval procedures. For the evaluation of such studies within the approval procedure, a preliminary framework for risk assessment was developed and should be further refined. It consists of a tiered approach that takes into account the outcome of all the assessments performed within this mandate.

Data gaps and uncertainties on the topics covered by the report were identified, alongside with respective recommendations on possible data collection/reporting activities and scientific studies, research, and scientific agreements needed to address them.

1 | INTRODUCTION

Among the infectious fungal species, *Aspergillus* spp., a filamentous fungus widespread in the environment, is one of the causative agents of the most serious fungal infections in humans. Aspergillosis, the infection caused by *Aspergillus* spp., occurs primarily in people with compromised immune systems or lung diseases. It gives a wide variety of clinical syndromes that include allergic reactions, pulmonary infections and infections in other organs. *A. fumigatus* is the most widely represented species, being the major cause of invasive aspergillosis (IA) with high mortality rates in immunocompromised patients (CDC, 2019; Garcia-Rubio et al., 2017; Rybak et al., 2019). Other important species include *A. flavus*, *A. terreus* and *A. niger*.

Azole medicinal products are indicated for the treatment of systemic fungal infections such as aspergillosis, as well as mycoses of the skin, mucous membranes and external genitalia. Azoles are available as intravenous, oral and topical formulations, for use according to the site of infection. Azoles are primary treatment options for aspergillosis, with four compounds available as medicinal products: itraconazole, voriconazole, posaconazole and isavuconazole.

The emergence of azole-resistant *Aspergillus* spp. is diminishing the effectiveness of azole therapy of patients with aspergillosis. Azole resistance in *Aspergillus* spp. may develop in the host during azole therapy (patient route) but may also develop in the environment following exposure to azole fungicides (environmental route). There is growing evidence that, when exposed to azoles in the environment, *Aspergillus* spp. develop azole resistance mutations, which confer cross resistance to the medical azoles. The incidence of azole resistance from environmental origin has been increasing over the past decade and has been primarily reported in *A. fumigatus*. The characteristics of azole resistance originating from the environment include dominance of a limited number of azole resistance mutations and has been described in azole-resistant aspergillosis in patients who had not previously treated with azoles (azole-naïve patients).

Other than their use as human medicines, azoles are widely used as fungicides in agriculture (for the control of fungal diseases in plants), as biocides (in particular as wood preservatives), as veterinary medicines (to treat fungal diseases in companion and food-producing animals), as industrial chemicals (e.g. as intermediates or dyes) and in cosmetics (e.g. as anti-dandruff agents or preservatives). Through such uses, azole fungicides may reach various environmental compartments. *A. fumigatus* is not a plant pathogen and therefore is not a target for the applications but, as all *Aspergillus* spp., it is a saprobic fungus that thrives on decaying plant material, where it may come in contact with residues of azole fungicides. Therefore, *Aspergillus* spp. can come in contact with azole fungicides from various sources and through various routes of exposure other than human medicinal products.

Several Member States have carried out studies or reviewed existing knowledge in this area, in particular looking at the conditions or uses which favour such azole resistance development in *Aspergillus* spp. (so-called environmental hotspots), and have raised concerns about the emergence of azole resistance from environmental origin, calling on the European Commission to further investigate the matter and consider any necessary regulatory actions or follow up work that may be required. For example, in Denmark and in the Netherlands (Member States with national programmes for monitoring of antifungal resistance in *Aspergillus* species)[1] environmental azole resistance mutations of environmental origin have been found in up to 74% and in 77%–84% (2016–2019) of ARAf isolates from patients respectively and represent an important cause of azole-resistant aspergillosis (Risum et al., 2020; Zhang et al., 2021).

Given those concerns, in March 2021 the Netherlands amended the authorisations of 12 azole-containing PPPs used on flower bulbs and tubers, including the flowers resulting therefrom, introducing a protocol to reduce the long-term storage of plant waste and reduce further development and spread of ARAf.

In 2013, the European Centre for Disease Prevention and Control (ECDC) published an initial technical report (ECDC, 2013) on this topic. Since then, further research has been undertaken and should now be reviewed to advance knowledge and understanding, and to provide the necessary information to inform on possible actions to limit the further emergence of azole-resistant *Aspergillus* spp. and its spread into humans. For this reason, and considering the One Health nature of this topic, the European Commission requested the European Food Safety Authority (EFSA), the European Chemical Agency (ECHA), the European Medicines Agency (EMA), the European Centre for Disease Prevention and Control (ECDC) and the European Environment Agency (EEA), with the scientific support from the European Commission's Joint Research Centre (JRC), to collaborate and produce a scientific report addressing the request further outlined in Section 1.1 below.

1.1 | Background and Terms of Reference as provided by the European Commission

1.1.1 | Background

The complete background to the request provided by the European Commission is publicly available on Open EFSA.[2]

[1] See also: https://swab.nl/en/nethmap-pvid369; https://www.ssi.dk/aktuelt/nyheder/2019/de-forste-tal-fra-den-nye-overvagning-af-resistens-i-svampen-aspergillus.
[2] https://open.efsa.europa.eu/questions/EFSA-Q-2022-00040.

1.1.2 | Terms of Reference

In view of the One Health nature of this subject, a scientific report was requested under Article 31 of Regulation (EC) No 178/2002 (for EFSA), Article 75 (1)(g) of Regulation (EU) No 528/2012 (for ECHA), Article 5(3) and Article 30(3) of Regulation (EU) No 726/2004 (for EMA) and Article 7(1)(a) of Regulation (EC) No 851/2004 (for ECDC). EEA was requested to contribute to the task by providing data, where available. The JRC has agreed to provide support to the task, including some experimental work.

Taking into account the background information provided and other available sources, the scientific report should address the following points regarding the development of azole resistance:

- provide details about the use of azole fungicides, other than as human medicines, in the EU/EEA giving information about the types of use, the trend in quantities used and as much detail as possible on geographical variation; ***[term of reference 1]***
- to what extent the current information suggests or supports the possibility of a causative link between the use of azole fungicides, other than as human medicines, and the development of azole resistant *Aspergillus* species. Experimental work carried out by the JRC to strengthen the available data on development of resistance should be taken into account; ***[term of reference 2]***
- in case a link is confirmed between exposure to azoles (in the environment) and development of resistance (point above):
    - a consideration of the epidemiology of azole resistant human infections caused by *Aspergillus* species acquiring resistance in the environment should be provided, considering patient exposure and transmission routes, in particular aerial dispersal; ***[term of reference 3]***
    - where the possibility of a significant risk to human health is identified, the risk to health should be quantified as far as possible in terms of levels of morbidity/mortality (including from occupational exposure e.g. to workers involved in agriculture or waste processing) now and in the future, in particular assessing the current scale of the problem and its relevance and impact on human health (and animal health, if relevant) in the EU, as well as an estimation of how the situation could develop in the future; ***[term of reference 4]***
- review the drivers/selection dynamics behind the development of environmental resistance in *Aspergillus* (in so called "hotspots"), in particular identification of specific types of uses, individual classes of substances, and the use conditions that lead to the development of environmental resistance (including conditions during storage and processing of (waste) materials with azole residues), providing, if possible, an indication of the selection risk from different uses and the contribution of sectoral uses of azoles. Experimental work should be considered in order to provide additional information on this point. ***[term of reference 5]***

In addition, taking into account the above, the agencies and JRC should provide advice on to what extent further actions by the EU could be considered to reduce any risks to human health identified and if so what those actions may be in view of providing the agencies, Member States and/or the Commission with information that could be taken into account during the assessment and decision-making of azole substances and/or products containing them. Where relevant sectorial specific advice should be included. In particular the following points should be considered:

- identify measures that could be implemented with respect to the use of azole fungicides and storage/processing of (waste) materials with azole residues and metabolites to prevent or minimise the development of environmental resistance or minimise the spread of resistant *Aspergillus* into patients; ***[term of reference 6]***
- identify the type of studies that could be provided by applicants when submitting applications for approval of azole substances for use other than as human medicines which would help to determine the risk of inducing mutations conferring resistance to medicinal azoles and which methodology should be used to assess that risk; ***[term of reference 7]***
- identify any uncertainties and further research needs that will provide information to inform decision-making for azole substances and/or products containing them at EU and national level. ***[term of reference 8]***

The agencies and JRC should endeavour to cover all the available scientific evidence in their assessment, including search and analysis following systematic literature review methodology.

1.2 | Interpretation of the Terms of Reference

Several aspects of the terms of reference (ToRs) above were clarified with the requestor of the mandate, i.e. the European Commission (EC), as explained below. Subsequently, based on these clarifications, specific assessment questions (AQ) were formulated to address the ToRs.

1.2.1 | Term of reference 1 ('*Use of azole fungicides*')

In relation to ToR 1 of the request (addressed in detail within Annex A of this report), it was clarified that:

- ToR1 should investigate and report all types of uses for which azole fungicides/antifungals are regulated and approved for use in the EU/EEA other than as human medicines, i.e. as plant protection products (PPPs), biocides (BPs), industrial chemicals and veterinary medicinal products (VMPs).
- Substances in the remit of ToR1, and of the mandate in general, are substances that:
  - are currently approved for use in the EU;
  - were approved in the past but are no longer approved in the EU;
  - have never been approved in the EU but that are known to be used in other regions and that might be (re)submitted for approval in the future.

  Substances previously approved in the EU are potentially relevant because they may persist in the environment and remain as sources of selection pressure even when no longer approved. Substances that have never been approved are also potentially relevant in the context of future applications, since approval might be requested. In both cases, although unlikely, use may also occur in the context of derogations, such as emergency authorisations of plant protection products.

- In order to limit the scope of ToR 1, and of the mandate in general, to substances for which evidence exists about the possibility to select for cross-resistance with medical azoles, to prioritise the substances considered and to limit the timelines needed for the data collection and its subsequent assessment, additional specific criteria were established to restrict the type of substances assessed to the most relevant ones for the development of resistance in *Aspergillus* spp.:
  - in particular, for ToR 1, and the mandate in general, only azole fungicides/antifungals which are part of the azole family, i.e. triazoles and imidazoles are included;
  - other compounds, for example substances with the same mechanism of action (sterol biosynthesis inhibition) such as piperazines, pyridines and pyrimidines, are considered outside the scope of this scientific report as there is lack of scientific evidence linking the use of those chemical families with the development of resistance in *Aspergillus* spp. They were however included in the systematic literature search to check for any scientific evidence linking the use of piperazines, pyridines and pyrimidines with the development of resistance in *Aspergillus* spp. (addressed within ToR 2, see Annex B);
  - azoles categories that may have other functions (e.g. azoles herbicides) are excluded from ToR1, and from the mandate in general.

- Notwithstanding the points above, possible inclusion of few additional substances in the scope of the data collection performed within ToR 1, or for possible experimental studies carried out by JRC on the topic (see Annex G), and of the mandate in general, might be considered as appropriate.
- The data collection on the above-defined uses of azole fungicides performed within ToR 1 would be limited to years 2010–2021, with the aim of focusing on the years when data are more likely to be available from most EU countries, while keeping a sufficiently long time period to observe possible trends or variations in sales or use. Chemicals can reach surface waters through a variety of routes, with major ones for pesticides and biocides being runoff from land and discharges from urban wastewater treatment plants. Under the EU Water Framework Directive, which sets standards for a limited number of harmful chemicals in surface waters, a 'watch list' has been established to gather information on concentrations of emerging pollutants. Ten azole compounds were added to the list as of 2020.[3] Although once a substance is in the water it is not usually possible to identify its original use, it would be of interest to report the results of the monitoring carried out in water, as well as making any relevant recommendations in relation to further monitoring that might be useful. Therefore, it was agreed to report the related information available in addition to the data on use of azole fungicides.

Further details on the selection of the substances considered for the scope of ToR1, and of the mandate in general, are included within Annex A.

Taking into account the aim and the above clarifications, the following AQs were formulated to address ToR 1:

- **AQ 1.1:** What are the most relevant azole fungicides regarding the possibility to cross-select for resistance to medical azoles, and to be therefore considered within the scope of this scientific report?
- **AQ 1.2:** What are the quantities of the selected azole fungicides sold/used in the EU/EEA from 2010 until 2021 as plant protection products, biocides, industrial chemicals and veterinary medicines, divided where possible by Member State and/or region, year and type of use?

[3]See Commission Implementing Decision (EU) 2020/1161 and Commission Implementing Decision (EU) 2022/1307.

1.2.2 | Term of Reference 2 *('Azole resistance mechanisms, and link between use of azole fungicides and azole-resistant Aspegillus spp. infections in humans')*

In relation to ToR 2 of the request (addressed in detail within Annex B of this report), it was clarified that it should aim to identify the presence of a link between the use of azole fungicides, other than as human medicines, and the development of azole resistant *Aspergillus* spp. by:

- compiling current evidence on the resistance mechanisms to azole fungicides present in *Aspergillus* spp. isolates from the human, agricultural, environmental and animal domains (see definition of the different domains in the *Glossary*);
- listing the most important hotspots identified in the scientific literature for the development of resistance to azole fungicides in *Aspergillus* spp. in the environment due to use of these substances other than for human medicine;
- concluding on the possible association between using azoles other than human medicines and human infections due to azole-resistant *Aspergillus* spp.

Taking into account the aim and the above clarifications, the following AQs were formulated to address ToR 2:

- **AQ 2.1:** Are azole resistance mechanisms present in *Aspergillus* infections in azole-naïve humans? If yes, what are these mechanisms, their frequency of occurrence and their geographical distribution?
- **AQ 2.2:** Are azole resistance mechanisms present in *Aspergillus* species isolates from food-producing and/or companion animals? If yes, what are these mechanisms, their frequency of occurrence and their geographical distribution?
- **AQ 2.3:** Are azole-resistant *Aspergillus* species isolates and/or mechanisms found more often in animals treated with/exposed to VMPs containing azoles than in azole-free animals?
- **AQ 2.4:** Are azole resistance mechanisms present in *Aspergillus* species isolates from agricultural sources? If yes, what are these mechanisms, their frequency of occurrence and their geographical distribution?
- **AQ 2.5:** Are azole-resistant *Aspergillus* species isolates and/or mechanisms found more often in agriculture settings and products thereof where azole fungicides are used than in azole-free agricultural products and settings?
- **AQ 2.6:** Are azole resistance mechanisms present in *Aspergillus* species isolates in environmental samples? If yes, what are these mechanisms, their frequency of occurrence and their geographical distribution?
- **AQ 2.7:** Are azole-resistant *Aspergillus* species isolates and/or azole resistance mechanisms found more often in samples from environments where azole fungicides are used than from azole-free environments?
- **AQ 2.8:** Are identical/clonal azole-resistant Aspergillus species strains present in different domains? If yes, which are these strains, their frequency of occurrence and their geographical distribution?

1.2.3 | Term of Reference 3 (*'Epidemiology of human infections'*)

In relation to ToR 3 of the request (addressed in detail within Annex C of this report), it was clarified that it should aim to compile current evidence on the epidemiology of human infections caused by azole-resistant *Aspergillus* species with resistance mechanisms typically associated with the environment, agricultural or animal domains (see definition of the different domains in the *Glossary*) considering patient exposure and transmission routes.

Taking into account the aim and the above clarifications, the following AQs were formulated to address ToR 3:

- **AQ 3.1:** Among all patients diagnosed with aspergillosis, what is the proportion of infections with azole-resistant *Aspergillus* species?
- **AQ 3.2:** Among all patients diagnosed with aspergillosis due to azole-resistant *Aspergillus* species, what is the proportion of azole-naïve patients?
- **AQ 3.3:** Is transmission from food-producing and/or companion animals to humans a likely transmission route for azole-resistant *Aspergillus* species?
- **AQ 3.4:** Is transmission from agriculture sources to humans a likely transmission route for azole-resistant *Aspergillus* species?
- **AQ 3.5:** Is transmission from environmental sources to humans a likely transmission route for azole-resistant *Aspergillus* species?
- **AQ 3.6:** What are the risk factors for infection with azole-resistant *Aspergillus* species?

1.2.4 | Term of Reference 4 (*'Risk assessment for human health'*)

In relation to ToR 4 of the request (addressed in detail within Annex D of this report), it was clarified that, in case a link is confirmed between exposure to azoles (in the environment) and the development of resistance, where the possibility of a significant risk to human health is identified, the health risk should be quantified as far as possible in terms of levels of morbidity/mortality (including from occupational exposure, e.g. to workers involved in agriculture or waste processing) now and in the future.

In addition, it was clarified that when addressing ToR 4, given the closeness of the topic, it would be appropriate to consider and discuss the possible management measures that could be implemented to prevent or minimise the spread of azole-resistant *Aspergillus* spp. from the environment to human patients, although this was originally included in ToR 6.

Relevant information on azole-resistant *Aspergillus* spp. in animals is reviewed and assessed within terms of reference 2 and 3, and such information is reviewed within ToR 4 with a view of assessing the risk posed to human health by isolates of animal origin. Despite azole resistance aspergillosis has been identified also in animals, where it can also represent a serious clinical condition, prevalence in animals is largely unknown, and therefore the assessment performed within ToR 4 was restricted to the impact on human health.

Taking into account the aim and the above clarifications, the following AQs were formulated to address ToR 4:

- **AQ 4.1:** What is the current risk and its relevance to human health in the EU/EEA?
- **AQ 4.2:** What prevention and control measures are available or could be explored to prevent or control the spread of azole-resistant *Aspergillus* spp. from the environment to human patients?

1.2.5 | Term of Reference 5 ('*Environmental hotspots*')

In relation to ToR 5 of the request (addressed in detail within Annex E of this report), it was clarified that:

- Information related to substances used and types of uses, will be collected and reported under ToR1.
- Information and conclusions from studies available in the scientific literature on the identification of hotspots for resistance development will be collected with an extensive literature search within ToR 5, criteria will be further defined for identifying specific environments that should be considered as environmental hotspots for development of resistance to azole fungicides in *Aspergillus* spp.
- ToR 5 requires listing and assessing the risk factors that influence the selection of resistance to azole fungicides in *Aspergillus* spp. within the environmental hotspots identified. If possible, the relative importance of the different risk factors should be investigated.
- In addition to information retrieved from scientific literature and research results, additional experimental evidence might be produced by JRC during the course of the mandate. Any results obtained will be included among the evidence considered to draw conclusions for this ToR.
- The evidence collected through the literature searches carried out to address this ToR as well as ToR 2 and ToR 3 highlighted that the main *Aspergillus* species concerned by the emergence and spread of azole resistance is *A. fumigatus*, while there is a lack of data related to other *Aspergillus* species. Therefore, the subsequent assessment carried out within this ToR focused on *A. fumigatus*.

In order to investigate the drivers and dynamics behind the selection of resistance in *Aspergillus* in the environment in so-called 'environmental hotspots' and the use conditions that lead to environmental resistance, the following AQs were formulated to address ToR5:

- **AQ 5.1**: What are the drivers and selection dynamics behind the development of resistance in *A. fumigatus*?
- **AQ 5.2:** Which applications of azoles may lead to ecological selection of ARAf strains?

1.2.6 | Term of Reference 6 ('*Prevention and control options*')

In relation to ToR 6 of the request (addressed in detail within Annex E of this report), it was clarified that:

- As mentioned above (Section 1.2.4), possible management measures that could be implemented to prevent or minimise the spread of azole-resistant *Aspergillus* spp. from the environment into patients will be covered when addressing ToR 4.

Taking into account the above clarifications, the following AQ was formulated to address ToR6:

- **AQ 6.1**: What prevention and control measures are available or could be realised to prevent creating environmental hotspots with selective advantage of ARAf?

1.2.7 | Term of Reference 7 ('Studies by applicants')

In relation to ToR 7 of the request (addressed in detail within Annex F of this report), it was clarified that:

- In relation to the types of studies that could help to identify the 'risk' for different azole substances to lead to cross-resistance to medical azoles, it was noted that the findings obtained when addressing ToR 2 would be relevant to identify

resistance mechanisms/mutations in *Aspergillus* spp. from environmental sources, e.g. TR_{46} and TR_{34}, which have been found of importance in the treatment of human infections. In addition, experimental work carried out by JRC in connection to this mandate could identify suitable studies to investigate the capacity of different azole substances to select that resistance.
- It was noted that studies to investigate the capacity of azole substances both to induce mutations and to select for existing resistant *Aspergillus* spp. in the environment would be relevant.
- It was considered that the findings obtained when addressing ToR 2 and ToR 3 (in respect of potential transmission routes from the environment to humans) and ToR 5 (drivers/selection dynamics behind the development of environmental resistance) could be important when considering the types of studies or data needed to evaluate the selection pressure and potential for human exposure to resistant *Aspergillus* spp. due to different product types according to their conditions of use in the field.
- Sector-specific EU legislation sets out data requirements for applications for approval of substances, including azoles, for use as biocidal products, plant protection products, veterinary medicinal products and industrial chemicals. These requirements include data to allow evaluation of risks to human and animal health, although the risk arising from the development of resistance to medically important azoles may not be specifically mentioned. It was considered that any already existing regulatory framework or relevant guidance on data requirements and risk methodology developed for individual sectors should be taken into account when responding to ToR 7. However, the recommendations did not need to be limited to the existing frameworks and could bring forward suggestions for future amendments.
- It was noted that although several published methodologies exist for assessing the public health risk associated with exposure to antimicrobial resistance through the food chain, particularly relating to use of pharmaceuticals (Caffrey et al., 2019; Codex Alimentarius, 2011), little has been published on methodologies for assessment of the AMR risk relating to exposure to chemicals via the environment (ANSES, 2019; FAO, 2019; Murray et al., 2021).
- For certain sectors/product types, the level of evidence for the risk and availability of validated studies may hinder the ability to make recommendations for regulatory requirements at this time.

Taking into account the above clarifications, the following AQs were formulated to address ToR7:

AQ7.1: What types of studies can be used to investigate the capacity of different azole substances to induce or select for resistance to medicinal azole substances in *Aspergillus* spp.?

AQ7.2: What types of studies or data can be used to determine the probability ('risk') for azole substances/products to select resistance to medicinal azole substances in *Aspergillus* spp. according to their conditions of use?

AQ7.3: Which methodology and studies or data should be used to assess the risk of development of resistance to medicinal azole substances in *Aspergillus* spp. in the context of applications for approval/authorisation of azole substances/products?

1.2.8 | Term of Reference 8 (*'Data gaps and recommended actions'*)

In relation to ToR 8 of the request (addressed in detail within a dedicated section within Annexes A–F of this report), it was clarified that:

- The ToR requests to identify and list the most important data gaps on the aspects covered by all the previous terms of reference, and in particular those that limit the level of certainty in the assessment of the risk to human health posed by the development of resistance in *Aspergillus* spp. following to the use of azole fungicides other than as human medicines, and in the assessment of the efficacy of related prevention and control measures.
- In addition, the ToR requests to identify the priority data collection and research needs that would allow to gather new data to reduce such gaps.

Taking into account the above clarifications, the following AQ was formulated to address ToR 8:

AQ 8.1: 'What are the most important data gaps identified in the areas covered by all the previous terms of reference?'

AQ 8.2: 'What are the most important data collection and research needs recommended to reduce the identified data gaps?'

1.2.9 | Experimental studies by JRC

During the preparation of the mandate by the European Commission, the JRC agreed in providing support to the work of the European Agencies in addressing the ToRs by performing some experimental work related to the development of resistance to azole fungicides in *Aspergillus* spp. It was agreed that the results obtained by JRC within the deadline for delivery of the current report will be included in this report (see Annex G) and considered in the assessment to the extent possible.

2 | APPROACH TO ANSWER THE TERMS OF REFERENCE AND STRUCTURE OF THIS REPORT

As requested by the European Commission, and due to its multidisciplinary nature, this mandate was addressed jointly by the five agencies (EFSA, ECDC, ECHA, EEA and EMA), with the contribution of JRC. Several interdisciplinary working groups were set up depending on the topic covered by the specific ToRs, involving expertise within the remit of the different agencies. The different ToRs presented above are interlinked. The outcome obtained when addressing specific ToRs was useful to other parts of the assessment, as explained below and summarised in Figure 1.

As requested by the mandate within ToR 1 (‘*use of azole fungicides*’), information was gathered in relation to the use of azole fungicides other than as human medicines in the EU/EEA, including therefore use as PPPs, BPs, industrial chemicals and VMPs. As anticipated in Section 1.2.1 above, and further explained in Annex A, a list of relevant azole substances (fungicides/antifungals) was defined, resulting in 36 PPPs, BPs and VMPs substances and 17 industrial chemicals. For these substances data related to sales/use were collected by means of a structured survey submitted to relevant EU/EEA national competent authorities for PPPs, BPs and VMPs, and by extraction of data from the International Uniform Chemical Information Database (IUCLID) for industrial chemicals by ECHA. The methodology used to collect and analyse the data is explained in detail within Annex A, which addresses the request formulated within ToR 1. The information collected within ToR 1 was used to provide an overview of the quantities of azole substances used over time and space in the EU/EEA countries in 2010–2021, and was also used as input when addressing ToR 2 and ToR 5 as shown in Figure 1 and explained further below.

In addition to the above data, requested by the mandate, data are presented also in relation to:

- the use of azole antifungals in human medicine, as collected by ECDC;
- the presence of azole compounds in surface water and ground water, as collected by EEA.

Both ToR 2 (‘*resistance mechanisms and link use-infections*’) and ToR 3 (‘*epidemiology of human infections*’) were addressed by means of extensive literature searches, aimed at retrieving relevant information from scientific literature.

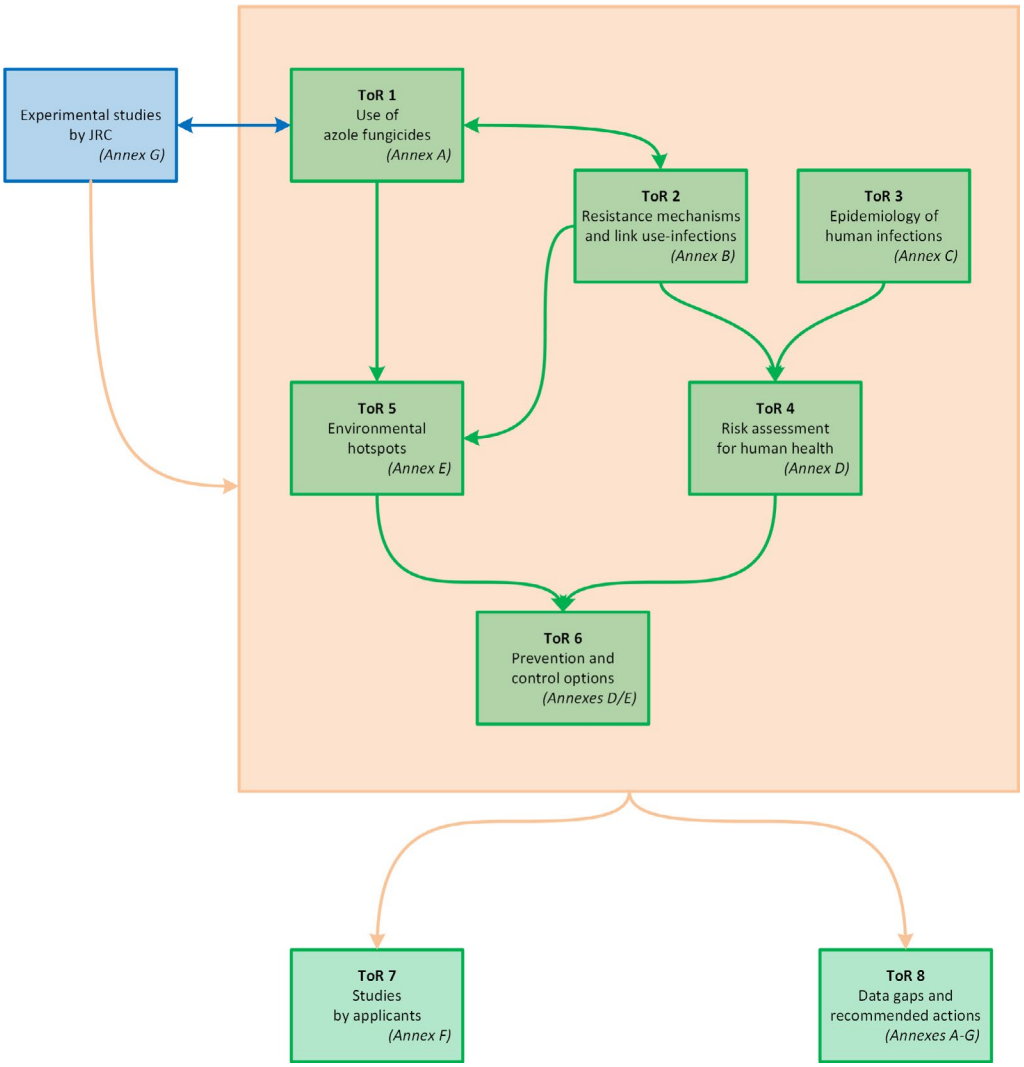

FIGURE 1 Interrelation between ToRs of the mandate (see text for explanation).

In particular, the findings related to ToR 2 are gathered within Annex B, compiling current evidence on the mechanisms of resistance to azole fungicides present in *Aspergillus* spp. isolates from the human, agricultural, environmental and animal domains. It also assesses the possible association between the use of azoles (other than as human medicines) and human infections due to azole-resistant *Aspergillus* spp. The literature search was informed by ToR 1, since all the relevant substances included in the ToR 1 survey were also explicitly used as search terms to retrieve scientific papers in the search performed. Conversely, the results of the literature search were used to identify any additional substance with evidence for resistance selection, which was then added to the survey on the sales/use carried out within ToR 1.

Findings related to ToR 3 are gathered within Annex C, compiling current evidence on the epidemiology of human infections caused by azole-resistant *Aspergillus* spp. with azole resistance mechanisms typically associated with the environment, agricultural or animal domains. It also discusses patient exposure and transmission routes.

The evidence gathered through the literature searches carried out to address ToR 2 and ToR 3 was also used to inform an expert elicitation exercise, addressing ToR 4 ('*risk assessment for human health*'), which aimed at evaluating the strength of such evidence and assessing the risk of the impact of using azoles outside the human sector and its implications for human health. The assessment employed a Delphi method, with different rounds of questions and discussion, to facilitate consensus building among the panel of experts involved. The methodology is further described, together with the outcome of the exercise, within Annex D.

Given the recognised role of the use of azoles other than as human medicines in the development of resistance to azoles in *Aspergillus* spp., as requested by the mandate, it was important to investigate the conditions that allow the selection of resistance in the environment and the most critical combinations of azole fungicides and specific uses leading to resistance selection, i.e. the so-called environmental hotspots. This was addressed by ToR 5 ('*environmental hotspots*'). Different methodologies were developed and used depending on the different types of regulated uses. Data gathered from different sources were used to inform the assessment, including (a) data on residues of azole fungicides in several products gathered from national databases, (b) data on the azole fungicides/antifungals used in the different environments collected within ToR 1 and (c) data from scientific literature reviewed within ToR 2 or identified through an additional literature search. The methodology and outcome of the assessment related to ToR 5 are reported in detail within Annex E.

ToR 6 ('*prevention and control options*') asked to identify and assess possible prevention and control options management options that would help in the prevention and control of the development of environmental resistance and its spread of resistant *Aspergillus* spp. into patients. These options were identified both from the literature searches carried out within the above ToRs and by expert opinion, leading to the formulation of several recommendations. Such recommendations are related on one side to the prevention and control of the selection of resistance in the environment following to the different types of use, which is addressed within Annex E, and on the other side to the prevention and control of the spread of resistance to patients, which is addressed within Annex D.

The mandate, within ToR 7 ('*studies by applicants*'), also requested to identify the type of studies that could be provided by applicants when submitting applications for the approval of azole substances for use other than human medicines, in order to be able to assess the possible risk for resistance selection. To formulate related recommendations, relevant information gathered through all the previous ToRs was taken into account. In particular, consideration was given to the relative quantities of azoles under the different regulatory frameworks, the information gathered through the literature on the main resistance mechanisms identified, the type of studies performed and the methodologies available to investigate them, as well as the outcome of the assessment of the different hotspots for resistance development and respective methodologies employed. The outcome of this exercise, with respective recommendations, is reported in detail within Annex F.

In addition to the data sources mentioned above (literature searches, various databases, expert opinion, etc.), some experimental studies were conducted by JRC in the timeframe of this mandate. These studies, which are reported in detail within Annex G, aimed at investigating the susceptibility of *A. fumigatus* exposed to triazoles and imidazoles, as well as some outlier compounds. Clinical, soil and compost material samples were used in the study to provide valuable insights on how these substances influence the incidence of antifungal resistance. Results from the studies obtained during the course of the mandate were shared with the EU agencies involved and were used to inform the expert opinion to answer some of the questions and to gather confirmations on the selection of substances considered in the survey carried out within ToR 1.

For each of the topics addressed above, several uncertainties and data gaps were recognised, which led to the formulation of recommendations for future research needs, as requested within ToR 8. This is discussed within each of the Annexes, depending on the topic. In addition, a summary of the macro-areas identified as gaps and recommendations for future research are provided in Section 3.9 of this report.

Within Section 4 of this report the main findings and conclusions of the different Annexes mentioned above are integrated and reported. The different Annexes will however need to be consulted to have a detailed explanation of the data and methodologies used, of the assessments performed, their related conclusions and, where relevant, recommendations.

3 | ASSESSMENT

This section reports a summary of the main findings, conclusions and recommendations of the different ToRs of the mandate, extracted from the information reported within the different Annexes to the report. Annexes A-G should be consulted for a detailed explanation on the data and methodologies used, for the results of the assessments performed, and complete conclusions and recommendations related to the different ToRs.

3.1 | Use of azole fungicides (answering to ToR 1)

This section reports the main findings and conclusions of Annex A, answering ToR 1 of the mandate. Please refer to Annex A for more detailed and complete information.

3.1.1 | Collection of data on use of azole fungicides

To answer the request formulated in ToR 1 of the mandate, data were collected from the competent authorities that were able to provide data in order to obtain information about the use of azole fungicides/antifungals, other than as human medicines, in the European Union/European Economic Area (EU/EEA). This data includes information about the types of use, the current status and trend in quantities used, and as much detail as possible on geographical variation.

A list of 131 azole fungicides used other than as human medicines was compiled (Annex A - Supplementary material 3 – Different lists of substances – Table 1). It includes active substances:

- Plant Protection Products (PPPs) regulated under Regulation (EC) No 1107/2009,
- Biocidal Products (BPs) regulated under Regulation (EU) No 528/2012,
- industrial chemicals regulated under Regulation (EC) 1907/2006 on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and
- antimycotics /antifungals – Veterinary Medicinal Products (VMPs) regulated under Regulation (EU) 2019/6.

This list consists of substances and their salts. The substances belong mainly to the triazole and imidazole chemical groups. For collecting data on use quantities and geographical variations, this list has been simplified, to assure that the collection of data was manageable and that the data were relevant. The salts were merged with the 'parent substances and substances with different identifiers were combined. Then only active substances which were approved between 2010 and 2021 at least under one of the regulations related to PPPs, BPs, VMPs in the EU or registered under REACH were chosen for collecting data. This resulted in 36 PPPs, BPs and VMPs substances (Annex A - Supplementary material 3 – Different lists of substances – Table 2) and 17 industrial azole chemicals (Annex A - Supplementary material 3 – Different lists of substances – Table 3) for which data were collected. Substances both approved and not approved (at the moment of the collection of the data) in the EU/EEA were included.

Monitoring data for concentrations in surface and groundwaters, reported to the EEA, were investigated for 17 substances.

Types of uses of azoles, quantities and geographical variation throughout the EU/EEA were collected from the Competent Authorities (Member State Competent Authorities, National Competent Authorities). To establish the quantities, the sales data were used as an approximation. The period used for reporting is 2010–2021. Data from the United Kingdom (UK) was neither requested nor used in the survey, as at the time of data collection, the UK was no longer member of the European Union.

The following limitations of the data collection should be noted:

- In relation to PPPs, it is important to note that due to the limitations in collecting data as a result of the provisions of Regulation (EC) No 1185/2009 and the protection of confidential data at the level of individual Member States, some Member States refrained from providing data (Spain) or provided limited information (Italy, which provided no information on quantities; Lithuania and Poland, which provided partial information). Data from Spain, Cyprus, Iceland and Switzerland are not shown on the graphs. The limited data from Italy, Lithuania and Poland are shown but are incomplete in comparison to other countries.
- Completed responses on the other questions in the PPP survey were received from all EU countries (except Cyprus and Spain) and from Norway ($n = 26$).
- The sales data or data on quantities of active substances placed on the market in BPs and VMPs is not required to be collected by the BPR and VMP Regulation. Therefore, this information is generally not known for the substances regulated by BPR and VMP Regulation. The data collected in this report on the BPR and VMP were provided on a voluntary basis and are not complete.
- For BPs data on quantities were only received from Belgium, Croatia, Denmark, Finland, France, Norway, Portugal and Sweden. Completed survey responses for BPs were received from all EU/EEA countries except Cyprus. ($n = 29$).
- For VMPs, 20 countries provided data on volumes of azole substances, however not all countries for the whole period 2010–2021. Completed responses to the other questions were received from all EU countries (except Croatia and Cyprus) and from Iceland ($n = 26$).
- Based on the REACH data on industrial chemicals, it was impossible to establish the current quantities used and trends nor geographical variation of the uses. All the reported quantities are confidential because they are based on only one or two producers per substance and therefore no conclusion on current quantities per individual substances and trends can be made.
- For environmental concentration data reported to the European Environment Agency's WISE Water Quality database, including those under the EU surface water Watch List, data from 19 countries were available.

Taking into account the above, it should be therefore considered that the data on quantities are therefore only illustrative, do not represent completely the situation in the EU/EEA and do not have a statistical value.

3.1.2 | Reported quantities of azole substances used

Even when considering the above limitations, it is still possible to provide the observations below.

Overall, around 120,000 tonnes of azoles (subject to this report) were reported to be sold on the EU/EEA market between 2010 and 2021 (Figure 2). Most of the azoles are used as PPPs (more than 119,000 tonnes). Azole fungicides are used all around Europe. The five azole substances with the highest tonnages reported are: tebuconazole (37,000 tonnes), prochloraz and prothioconazole (around 20,000 tonnes each), and epoxiconazole and metconazole (each around 9000 tonnes). Out of these, prochloraz and epoxiconazole are no longer approved as active substances under PPP. This has led to a drastic decrease in quantities for these two substances from the time of expiry of approval.

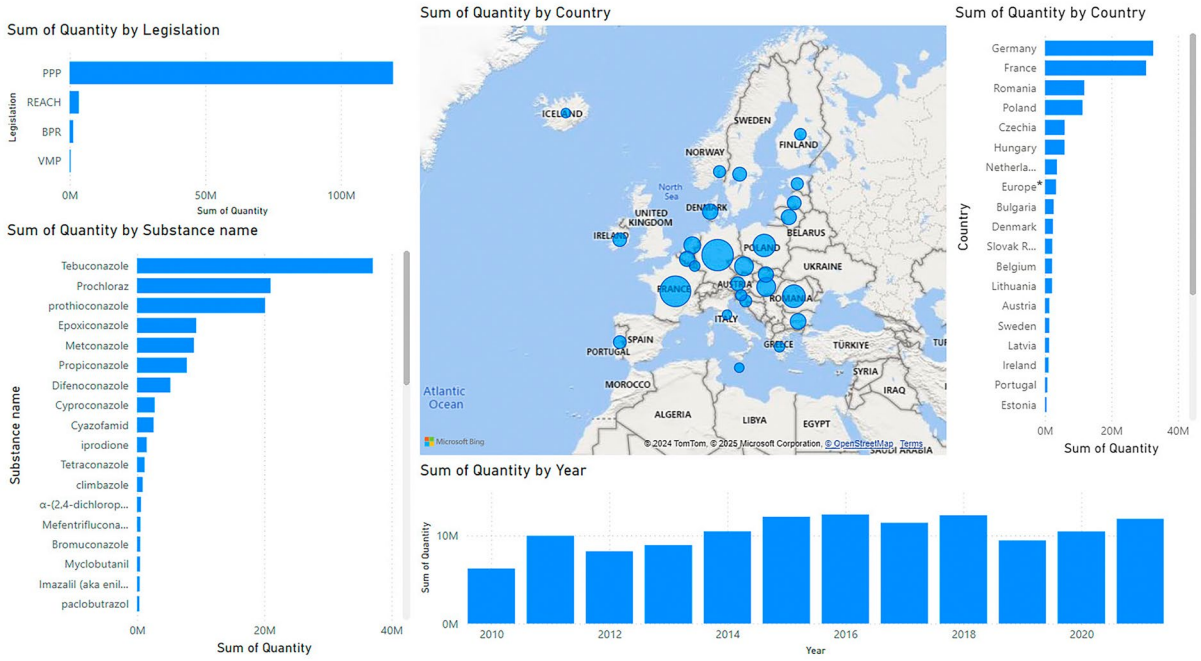

FIGURE 2 Overall sum of quantities of azoles reported as sold for 2010–2021 per Regulation (PPP, BPR, VMP, REACH), per substance, per country, per year and the geographical variation, please note the limitations of the submitted data. These data do not represent the complete situation in the EU/EEA. Europe*: aggregated data about industrial chemicals (REACH), for which no further geographical breakdown is available. Any designation of Kosovo is without prejudice to positions on status and is in line with United Nations Security Council Resolution 1244 and the International Court of Justice Opinion on the Kosovo Declaration of Independence.

Plant protection products

The trend of using azole fungicides is driven by PPPs in EU/EEA in the years 2010 to 2021 and is very stable, on average 10,000 tonnes a year.

Countries that have provided the data for PPPs, for the period 2010–2021 and reported the highest quantities are Germany and France, followed by Romania, Czechia and Hungary. As mentioned above, the limitation of this dataset is that due to the provisions of Regulation (EC) No 1185/2009 and the protection of confidential data at the level of individual Member States, some Member States refrained from providing data or provided limited information. Data from Spain, Cyprus, Iceland and Switzerland are not shown on the graphs. The limited data from Italy, Lithuania and Poland are shown but are incomplete in comparison to other countries.

Normalisation of data was only possible for PPPs, but not for BPs and VMPs, nor for industrial chemicals. For PPPs, after comparing the normalised (sales per hectare arable land and permanent crops) and non-normalised data, it can be assumed that the quantities of azole fungicides put on the market on a yearly basis can be directly associated with the use of those pesticides.

Following normalisation of the PPP data, it is concluded that the Netherlands and Germany are ranking in the first two top countries of azoles fungicides sales per hectare arable land and permanent crops (781 kg/1000 ha and 313 kg/1000 ha accordingly). Among all Member States surveyed, the top five EU countries reporting the highest sales volumes per hectare arable land and permanent crops are the Netherlands, Germany, Czechia, Ireland and Belgium. Among the top five countries having the highest pesticides sales, Germany and Czechia ranked also in the top five of pesticide sales per hectare

arable land and permanent crops. The sales for prothioconazole and tebuconazole have increased over the years and have been reported accounting for 58% of the total reported azole sales of PPPs.

The Netherlands, Germany, Czechia, Ireland and Belgium are ranked in top five countries by using both data on uses and on sales as provided by the industry association after normalisation. It is therefore assumed that the quantities of azoles fungicides introduced to the market yearly can be directly associated to the actual use of those pesticides.

Other uses, e.g. of propiconazole, have been decreased to zero due to the current status of the active substance (no longer approved as active substance under PPP). However, the substance is still allowed to be used under the BP Regulation and has also been registered under REACH.

Biocidal products

Azoles are highly important in wood protection as they are intended to destroy or control harmful or unwanted organisms such as fungi that have detrimental effects on wood and are used in a wide variety of ways by both industrial and professional users as well as by the public.

The overall amount of the four substances reported to be placed on the EU market between 2010 and 2021 is around 1200 tonnes. Most of the quantities (around 862 tonnes) belong to propiconazole, followed by tebuconazole (cca. 400 tonnes). Cyproconazole and imazalil were reported in quantities cca. 4 tonnes each. These data are considered underestimated, as only 28% of competent authorities provided quantities.

France reported most of the quantities followed by Sweden, Belgium and Denmark. Due to the limited number of respondents no trend in quantities can be concluded.

It was not possible to draw any firm conclusions regarding trends in sales of azole biocidal products since 2010. Azole biocidal products are used in all EU countries and Iceland, but no conclusions could be made about the level of use in different geographic regions.

Under the BPR, imazalil and cyproconazole are no longer allowed to be placed on the EU/EEA market.

Propiconazole was recently renewed as wood preservative (PT8) until 30 November 2030, with specific uses including temporary treatment against wood-discolouring fungi (anti-sapstain use through industrial treatment). For PT7 (film preservatives) and PT9 (fibre, leather, rubber and polymerised materials preservatives), there are no authorised products on the market. Propiconazole was reported to be used as wood preservative in all 29 countries.

Tebuconazole is in the process of renewal as wood preservative (PT8). Biocidal products containing tebuconazole were reported as placed on the market in 28 countries between 2010 and 2021. The only country in which tebuconazole was not on the market is Malta.

Veterinary medicinal products

Most of the azole antifungals present in VMPs are also used in human medicine. For instance, itraconazole is present in both veterinary and human medicine, where it is recommended in treatment guidelines as a first-line option for chronic pulmonary aspergillosis in humans. However, it is important to point out that its use in veterinary medicines is very limited; in particular, it is used to treat aspergillosis in ornamental birds.

Azoles are highly important in veterinary medicine being one of the few antifungal drug classes available to treat diseases that have significant impact on animal welfare and that in the case of dermatophytosis may be a zoonotic risk to public health.

The sales for imazalil/enilconazole, miconoazole (nitrate), clotrimazole, econazole and posaconazole make up 90% of the total reported sales of azole VMPs.

The total quantity of reported azole sales in VMPs in 19 EU countries plus Iceland over the entire period covered by the survey from 2010 to 2021 was 22.5 tonnes. Although it is difficult to extrapolate the actual EU sales, this is likely to represent < 0.02% of the sales across all four sectors, PPPs, biocides, chemicals and VMPs.

It was not possible to draw any firm conclusions regarding trends in sales of azole VMPs since 2010. Azole VMPs are used in all EU countries and Iceland, but no conclusions could be made about the level of use in different geographic regions.

Industrial chemicals

Based on the REACH data, we cannot establish the current quantities used and trends nor the geographical variation of the uses.

All the reported quantities are confidential because they are based on only one or two producers per substance and therefore no conclusion on current quantities per individual substances and trends can be made.

Substances widely used in personal care products like shampoos and therefore having wide dispersive use and a release into the environment, might need to be further investigated especially if having an antifungal function (e.g. anti-dandruff).

The case of propiconazole is particularly intriguing; its ban under the PPP Regulation coincided with a spike in its registration under REACH for the years 2020 and 2021. This could suggest a shift in the substance's market dynamics or regulatory categorisation, reflecting the adaptive nature of chemical manufacturing in response to regulatory changes. Such observations underscore the importance of robust and dynamic regulatory frameworks like REACH, which can accommodate

new data and adapt to evolving environmental and health concerns. The continuous monitoring and reassessment of substances ensures that regulatory actions remain relevant and effective in protecting human health and the environment.

Environmental occurrence (monitoring)

Analysis of concentrations of selected azoles in European surface and groundwaters has identified exceedance of aquatic safety thresholds in some surface waters, with fewer exceedances of the quality threshold in groundwater.

Environmental concentration data can rarely be attributed to a particular sector. Instead, they reflect the integrated effect of substance use.

The volumes of azoles used in human medicines and in agriculture are of similar total amount and so could be contributing to the observed concentrations in water to a similar degree. While this analysis does not allow source identification, typically urban wastewater treatment will break down some of the compounds, whereas runoff from agricultural use will not normally be subject to any treatment prior to entry into water courses.

Since water needs to percolate into groundwater, and once there, the residence time can range from years to decades, contamination of groundwater represents a concern, particularly if the resource is used for drinking water. The only substance found to exceed the quality threshold in groundwater was tebuconazole, perhaps reflecting the high amount of this substance applied in Europe. There is no EU requirement to measure tebuconazole in groundwater, so the actual level of contamination is uncertain for most countries.

The most frequent exceedance of the PNEC aquatic safety threshold was for tebuconazole. As a surface water watch list substance, all EU Member States should be reporting monitoring data. Of the 19 Member States that did report monitoring data for tebuconazole, 9 showed exceedances of the PNEC.

Exceedances of the PNEC for prochloraz occurred in the period 2018–2022, with some occurring also in 2022, after the approval for prochloraz use as a PPP was withdrawn. This may reflect use of stored pesticides or other reasons which are unclear at this time.

Overall comparison of the amount of azoles used as human medicines vs. other uses

To put the amounts of azole fungicides used in the EU/EEA into context, a rough comparison on the amounts of azole fungicides used in human medicine with the amounts resulting from the collection of their use under PPP, BPR, VMP, REACH was made. In 2021, the amount of triazole and tetrazole derivatives for systemic use consumed by humans (12.2 tonnes, including both the community and hospital sectors) was about 1000 times smaller than the amount of azole substances reported in PPP, BPR, VMP and REACH in 2021 (11,886 tonnes). There are methodological differences in the data collection and their representativeness for the EU/EEA population, so this comparison is only illustrative.

3.1.3 | Uses of the reported azole substances

There are many ways in which the checked azole substances are used throughout Europe:

- In PPPs they have been widely used to control fungal plant diseases in major crops, including cereals, oilseed rape, sugar beet, bananas, rice, soybeans, oranges and turfgrass particularly on golf courses etc. It should be noted that, while for PPP data are available on the sales amounts of specific products, data on the use of specific products/substances in the different crops are not available and can only be estimated based on instructions for use of the different products.
- In BPs the main use is as wood preservative.
- VMPs containing azoles are primarily formulated for topical administration to treat dermatophytosis and yeast infections on the skin and otitis externa in the ear canal being predominantly authorised for use in companion animals.
- As industrial chemicals the azole substances are mostly used as intermediates (precursors) to manufacture yet a different substance; they are reported to be manufactured as active substances in PPPs, BPs or VMPs or are formulated into a mixture. Only one substance – climbazole – has a widespread use. It is used as an anti-dandruff substance in cosmetics.

The substances with most types of uses overall reported between 2010 and 2021 are: tebuconazole (26 different uses), iprodione (25), difenoconazole (24) propiconazole (17) and cyazofamid (11). All of these are PPP substances, tebuconazole and propiconazole are also active substances in BPs.

The most used substance overall is tebuconazole (regulated under PPP and BP Regulations), with around 37,000 tonnes over the period 2010–2021. This represents 31% of all the azoles analysed in this report. The trend from 2014 is rather stable, close to 4000 tonnes per year.

It is used in PPPs to protect mostly (use reported by more than 10 Member States) cereals, cereal seeds, grapes, oilseed rape, ornamentals, pome fruits, stone fruits and in biocidal products as a wood preservative (Figure 3).

FIGURE 3 Tebuconazole uses reported by more than 10 EU/EEA countries. Any designation of Kosovo is without prejudice to positions on status and is in line with United Nations Security Council Resolution 1244 and the International Court of Justice Opinion on the Kosovo Declaration of Independence.

In BPs, tebuconazole and propiconazole are mostly used as wood preservatives. Propiconazole was recently (2024) renewed under BPR, but it's use is restricted to certain uses.[4] Since 2020 propiconazole is not allowed in PPPs.

For VMPs, miconazole (nitrate) accounts for 36% of total veterinary azole sales over the entire period, and it has been the azole with the highest reported sales in the last 2 years (2020 and 2021). VMPs containing miconazole are available in 25 countries (2021) and are exclusively presented as ear and cutaneous preparations for use in cats and dogs. Similarly, clotrimazole, econazole and posaconazole are also only available in VMPs as cutaneous or ear preparations.

The sales for imazalil/enilconazole, miconoazole (nitrate), clotrimazole, econazole and posaconazole make up 90% of the total reported sales of azole VMPs, indicating that the major route of administration of azoles in veterinary medicine is topical.

Analysis of concentrations of selected azoles in European surface and groundwaters has identified exceedance of aquatic safety thresholds in some surface waters, with fewer exceedances of quality thresholds in groundwater. Tebuconazole in surface waters was found to be above the aquatic safety threshold at monitoring sites in nine out of 20 countries that reported monitoring data to EEA and prochloraz in 3 out of 20 countries. Tebuconazole in groundwater exceeded the quality threshold at sites in 6 out of 10 countries that reported monitoring data to EEA.

No clear conclusion can be made on the geographical variation of uses. The substances are reported to be used in most of the countries without any clear trend to make a possible distinction of uses in the EU/EEA.

3.2 | Azole resistance mechanisms, and link between use of azole fungicides and azole-resistant *Aspergillus* spp. infections in humans (answering to ToR 2)

This section reports the main findings and conclusions of Annex B, answering ToR 2 of the mandate. Please refer to Annex B for more detailed and complete information.

Annex B compiles current evidence on the mechanisms of resistance to azole fungicides present in *Aspergillus* spp. isolates from the human, agricultural, environmental and animal domains. It also assesses the possible association between the use of azoles (other than as human medicines) and human infections due to azole-resistant *Aspergillus* spp. As described in Section 2 above, the information reported is based on the results of a specific extensive literature search. Most of the scientific literature reviewed reported evidence related to emergence and spread of azole resistance in *Aspergillus fumigatus* and azole-resistant *A. fumigatus* (ARAf), while there is a lack of data related to other *Aspergillus* species.

Two routes of exposure that may lead to the development of azole resistance in *Aspergillus* spp. isolates have been identified: (i) the patient-acquired route, resulting from long-term azole therapy and (ii) the environmental route, caused by the

[4] Implementing regulation - EU - 2023/2596 - EN - EUR-Lex (europa.eu).

use of azole fungicides in the environment. There is growing evidence that *A. fumigatus* may develop cross-resistance to medical azoles if exposed to azole fungicides in the environment.

Most reported resistance mechanisms of *A. fumigatus*, which may confer resistance to one or more azole fungicides, are linked to mutations of the *cyp51A* gene and its promoter. Mutations coding for azole resistance in *A. fumigatus* isolated from the environment are commonly composed of tandem repeats (TRs) in the promoter region of the *cyp51A* gene in combination with single or multiple-point mutations (e.g. TR_{34}/L98H, TR_{53}, TR_{46}/Y121F/T289A).

Despite growing evidence, further studies on environment-to-patient transmission routes are needed to evaluate the extent to which environmental sources of azole resistance in *A. fumigatus* are the dominant driver for azole resistance encountered in human *A. fumigatus* infections. The *cyp51A* mutations (e.g. TR_{34}/L98H) that are typical of *ARAf* isolates from the environment have been reported in *A. fumigatus* isolates in many countries worldwide. The most prevalent mutation in ARAf isolates from the environment was TR_{34}/L98H, followed by multiple-point mutations. Similarly, most studies from the agricultural domain identified the common TR_{34}/L98H and TR_{46}/Y121F/T289A mutations. Only three studies covered the animal domain: one on dogs and cats in Australia, another on birds and avian farms in southern China and France, and a recent one on clinical isolates from birds, cats, dogs and free-ranging harbour porpoise in the Netherlands.

The observations from the included studies showed that the most typical azole resistance mechanisms, i.e. specific tandem repeat mutations in the promoter region of the *cyp51A*, gene, which are common in ARAf isolates from environmental sources, are omnipresent in clinical ARAf isolates from humans. These mutations can be found in *A. fumigatus* isolates recovered from patients receiving azole therapy, as well as in azole-naïve patients, again suggesting the environmental route of resistance through exposure to azole fungicides in agriculture. The most frequently identified molecular resistance mechanism in *A. fumigatus* isolates from azole-naïve patients was the TR_{34}/L98H mutation, followed by the TR_{46}/Y121F/T289A mutation, as well as the G54E and Y121F mutations.

Patient-to-environment transmission of ARAf is considered rare, as *Aspergillus* spp. primarily spread through the release of conidia. This further supports the hypothesis that transmission of ARAf occurs from the environment to humans. Nevertheless, further research with the specific objective of confirming the directionality of inter-domain spread is required.

Multiple environmental sites have been reported as hotspots for the development and selection of ARAf: decaying flower bulbs and green waste, industrial wood chippings, wood from sawmills, and air and soil from hospital surroundings. This list is most likely not exhaustive and studies to identify possible additional hotspots and their relevance to infections caused by ARAf are needed. A specific assessment aimed at identifying environmental hotspots for resistance has been conducted in answer to ToR 5 of this mandate (see Section 3.5 and Annex E).

The rapid global spread of azole resistance has been observed since the emergence of TR_{34}- and TR_{46}-based *cyp51A* variants. Numerous studies have documented the clonal dissemination of strains with these types of molecular resistance mechanisms across clinical, environmental and agricultural settings. While azole resistance in *A. fumigatus* is exceptionally high in Europe, varying rates have been reported in other parts of the world. The international spread of ARAf isolates has been associated to the global trade of plant materials. Import of ARAf on Dutch plant bulbs has been identified in several countries as a route of environmental exposure to ARAf for patients at risk of ARAf infection. Studies have documented the spread of ARAf through bulb imports from the Netherlands to France, Ireland and Japan. Other agricultural commodities may have a similar role. The rapid global spread of clonal lineages of ARAf with TR_{34} and TR_{46} mutations (often in combination with other genetic alterations), as well as the risk of spread related to the importation and exportation of agricultural products from hotspots for ARAf, are of concern.

The identified and included studies used multiple designs, sampling strategies and sample sizes, with many studies aiming to investigate the molecular resistance mechanisms and mutations carried by ARAf in patients with aspergillosis. However, not all studies focused on azole-naïve patients and studies often lacked information on the patients' prior exposure to azole therapy; thus, their treatment status was unclear.

Further studies on the critical factors involved in the development, selection and spread of antifungal resistance in *Aspergillus* spp., on antifungal-resistant *Aspergillus* spp. isolates, and on the environment-to-patient transmission route would aid in the design and implementation of strategies to prevent and control aspergillosis caused by azole-resistant *Aspergillus* spp.

3.3 | Epidemiology of human infections (answering to ToR 3)

This section reports the main findings and conclusions of Annex C, answering ToR 3 of the mandate. Please refer to Annex C for more detailed and complete information.

Annex C compiles current evidence on the epidemiology of human infections caused by azole-resistant *Aspergillus* spp. with azole resistance mechanisms typically associated with the environment, agricultural or animal domains, and considers patient exposure and transmission routes. As described in Section 3 above, the information reported is based on the results of a specific extensive literature search. Most of the scientific literature reviewed reported evidence related to the epidemiology of infections with azole-resistant *A. fumigatus* (ARAf), while there is a lack of data on azole resistance related to other *Aspergillus* species.

The global spread of azole-resistant *Aspergillus* spp. is a public health concern.

While azole resistance in *A. fumigatus* infections is high in Europe, varying rates have been reported in other parts of the world. The reported prevalence of ARAf in human *A. fumigatus* infections varied among the different presentations of the disease:

- Invasive aspergillosis (IA) – between 0.7% and 63.6%, with the highest prevalence reported in India and the Netherlands, and in one centre in Germany.
- Chronic pulmonary aspergillosis (CPA) – between 5.9% and 59.2%, with the highest prevalence reported in France and India.
- Allergic bronchopulmonary aspergillosis (ABPA) – between 2.3% and 42.8%.

This variation can be attributed to various factors such as differences in antifungal exposure, environmental factors and the emergence of specific azole-resistant strains, but also due to detection bias in certain areas. It is important to interpret the reported prevalence of azole resistance in *Aspergillus* spp. and aspergillosis with caution, as the use of different denominators greatly impacts prevalence estimates.

Due to the low sensitivity of mycological cultures, many patients remain 'culture-negative' and aspergillosis is instead diagnosed through the detection of the galactomannan antigen, and antifungal susceptibility testing cannot be performed. If the prevalence of azole resistance is calculated based only on culture-positive cases or on *Aspergillus* spp. isolates, this may lead to an overestimation of the prevalence of azole resistance in aspergillosis. Therefore, a unified approach to reporting is necessary to compare azole resistance prevalence between countries and allow for developing and implementing targeted interventions where they are the most needed. In this context, detection of resistant genotypes with molecular methods directly from clinical samples has the potential to become a valuable tool to rapidly identify patients with azole-resistant aspergillosis.

Inversely, the prevalence of azole resistance in *Aspergillus* spp. may be underestimated due to the absence of routine antifungal susceptibility testing and the lack of harmonised/standardised protocols and interpretative criteria for susceptibility testing.

The small sample size of most studies reporting on the prevalence of azole resistance in *Aspergillus* spp. infections affects the accuracy of prevalence estimates. Multicentre studies should provide a more reliable estimate of azole resistance prevalence. Mutations responsible for azole resistance in environmental isolates, particularly the TR_{34}/L98H mutation, have been consistently reported in human infections and the TR_{46}/Y121F/T289A mutation has also been identified.

Cases of aspergillosis due to ARAf have been reported in azole-naïve patients, mostly in patients with IA. This rules out azole therapy as a route for selection of azole resistance and ARAf in these patients and underscores the role of other routes of azole resistance selection such as the environment (see Annex B). For patients with CPA and ABPA due to ARAf, the information about previous azole exposure is scarce.

While most studies on azole resistance prevalence focus on patients with invasive aspergillosis, continuous efforts should be made to monitor the emergence of azole resistance in other forms of aspergillosis, given the current global distribution of azole-resistant *Aspergillus* spp.

To develop effective strategies for preventing and controlling the spread of azole-resistant *Aspergillus* strains, it is crucial to understand the possible routes of transmission of azole resistance to humans. Some clinical infections have been linked to exposure to *Aspergillus* spp. from agriculture, animal farms, plants, wood mills, construction/demolition sites and the hospital environment, thus indicating the potential for occupational disease as well as the possibility of healthcare-associated outbreaks. However, due to the high genetic diversity of *A. fumigatus*, genotypic analyses did not always confirm the genetic relatedness between clinical and environmental isolates. The high rate of cross-overs per chromosome pair in *A. fumigatus* may limit the applicability of genotyping for this species. Additionally, some studies have been limited by the small number of environmental samples and of clinical isolates, which may have resulted in the absence of positive findings regarding the relationship or transmission between the environment and patients. In addition, given the high diversity of current typing methods, the role of WGS in studies or azole resistance transmission should be assessed.

There is a need for studies with high number of environmental samples and clinical isolates to further support this association.

While the use of antifungals in animals is described as limited, and aspergillosis is not regarded as a zoonotic disease, it would be important to assess azole resistance in this context, considering the potential contamination of the surrounding environment. Contamination of the environment by animals, namely in animal farms, has been described. However, the number of studies is limited. Animal-related contaminated environments may work as a possible route of transmission to humans, particularly in geographical areas with a higher prevalence of azole resistance in *Aspergillus* spp. Surveillance studies should be performed in a One Health perspective all settings where there is a high use of azole fungicides (e.g. also including agricultural areas and farms) and relatedness with human cases should be investigated. These studies should be coordinated at the European level.

There was inconsistency in the methods used to study the transmission of ARAf between various domains. Standardisation of surveillance and study methods is needed for a more reliable testing and detection of azole-resistant aspergillosis.

Besides prior exposure to antifungal therapy, especially with azoles, additional risk factors associated with aspergillosis due to ARAf, and more generally azole-resistant *Aspergillus* spp., have been identified. These include underlying immunosuppression, such as patients with hematologic malignancies or solid organ transplantation, chronic obstructive pulmonary disease, cystic fibrosis and chronic granulomatous disease, as well as immunosuppressive therapy. Long-term

hospitalisation, especially in regions with a high prevalence of azole resistance in *Aspergillus* spp. has also been described as a risk factor. Exposure to agricultural areas and occupational exposure, such as ground maintenance and woodwork, have also been identified as potential risk factors for the acquisition of azole-resistant *Aspergillus* spp.

IA due to ARAf is associated with poor outcomes, including dismal mortality rates compared to IA due to azole-susceptible isolates. Studies generally reported a high mortality rate associated with IA caused by ARAf, ranging from 36% to 100%. All-cause mortality was generally higher in patients with IA due to ARAf than in patients with IA due to azole-susceptible *A. fumigatus*., ranging from 13.5% to 60% higher. The patient populations varied between studies, either regarding underlying disease or admission to an intensive care unit. The antifungal treatment strategy used for these patients was variable, from monotherapy with azoles, sequential treatment with different antifungal classes or a combination of antifungal agents from different classes. No assessment of the impact of these different strategies on the outcome was found in the literature.

This raises concerns about the use of voriconazole, the first-line treatment for suspected IA, in patients who may have azole-resistant isolates. Nevertheless, current guidelines recommend voriconazole as the primary therapy if local azole resistance rates remain below 10%, with liposomal amphotericin B (L-AMB) as an alternative option. Delayed diagnosis and/or delayed initiation of effective therapy also contribute to poorer outcomes. The small number of infections due to ARAf included in some of the analyses, limits the assessment of azole resistance and its impact on mortality from IA. For CPA and ABPA due to azole-resistant *Aspergillus* spp., while the potential impact of azole resistance in patient management is increasingly being acknowledged, the number of studies reporting on the prevalence and outcomes of azole resistance in these patients is limited. This highlights the need for further research to determine the prevalence of azole resistance in CPA and ABPA, and identify associated risk factors. Such studies will be critical in improving patient outcomes and guiding clinical decision-making.

3.4 | Risk assessment for human health (answering to ToR 4)

This section reports the main findings and conclusions of Annex D, answering ToR 4 of the mandate. Please refer to Annex D for more detailed and complete information.

Annex D assessed the risk of the impact of using azoles outside the human sector and its implications for human health across various at-risk groups and geographic locations. As indicated in Section 2, the assessment was based on an expert elicitation exercise, employing the Delphi method to facilitate consensus building among the panel of experts involved. Across four Delphi rounds, the experts reached consensus on most aspects. However, the predominant theme throughout the exercise was uncertainty in decision-making which was primarily due to gaps in current research and available data.

The experts identified key risk factors and at-risk groups for infections with ARAf. In accordance with the results of the literature search described in Section 3.3 above, they highlighted uncertainties in assessing the prevalence of ARAf among all cases of human invasive aspergillosis due to *A. fumigatus* in the EU/EEA. Therefore, estimating the current prevalence of ARAf among all human cases of invasive aspergillosis due to *A. fumigatus* in the EU/EEA was hindered by an unsatisfactory level of confidence among the experts because available information mainly rely on several sentinel centres and single centre studies with various denominators. Moreover, cross-border dissemination of azole resistance in *A. fumigatus* in the EU/EEA is considered by the experts likely or very likely, although the current data are deemed insufficient to support this statement. Whole-genome sequencing can enhance the understanding of cross-border dissemination of ARAf isolates.

Azole usage outside the human domain is likely or very likely to contribute to the selection of azole-resistant *A. fumigatus* (ARAf) isolates that could cause severe disease such as IA, but the extent of this contribution needs to be better understood.

A call was made for the establishment of a shared interdisciplinary forum for stakeholders (e.g. clinical microbiologists, researchers, public health professionals) and the implementation of awareness-raising initiatives to comprehensively address the issue of strengthening diagnostic capacity and disease-specific surveillance, to prevent the selection and dissemination of azole-resistant *A. fumigatus*, after the expert panel stressed its importance (see also Section 3.6). Finally, recommendations included the incorporation of regulatory measures within the environmental and agricultural sectors, coupled with the adoption of a systems thinking approach to navigate the multifaceted complexities inherent to the problem.

3.5 | Environmental hotspots (answering to ToR 5)

This section reports the main findings and conclusions of Annex E, in relation to the specific parts of that Annex answering ToR 5 of the mandate. Please refer to Annex E for more detailed and complete information.

The aim of Annex E was to review the ecological selection dynamics driving the development and spread of environmental resistance in *Aspergillus* spp. (in so-called 'environmental hotspots'). In particular, the identification of specific types of uses, individual classes of substances and the use conditions were sought that lead to the development of environmental resistance including conditions during storage, processing and disposal of organic (waste) materials containing azole residues. An attempt was made to assess the selection risk from different uses and the contribution of sectoral uses of azoles and to identify research needs and the experimental work that should be carried out to provide additional information. Previous work reported in literature provided evidence for environmental azole resistance selection in *A. fumigatus*,

which are widespread in the environment. The work focused on the drivers and dynamics of ecological selection of those resistant *A. fumigatus* strains in the environment that would lead to an increase of resistance in the environment, the dispersal of resistant strains and potential exposure of humans.

Some hotspots for resistance selection have been already reported in scientific literature. Annex E aimed to identify further environments as environmental hotspots for resistance selection and to list and assess risk factors for the selection of resistant *Aspergillus* spp. when exposed to azole fungicides. Where possible, the relative importance of the different risk factors was investigated.

Given that the sales of VMP azoles constitute a small percentage (less than 0.02%) of the total azole sales in comparison to PPPs, BPs and industrial chemicals, and considering that VMPs are applied directly to targeted areas (typically treatment for skin lesions), their use is not directly comparable to that of PPPs and BPs which are typically dispersed into the environment. Consequently, the current report focused on the use of PPPs, BPs and industrial chemicals for the development of environmental hotspots.

From an extensive list of azole fungicides, 36 substances were shortlisted for collection of data on sales quantities and uses in the EU/EEA countries (see Annex A for details). To identify favourable growth conditions for *A. fumigatus* for establishing azole-resistant environmental hotspots, an extensive literature search was performed.

Of the 36 substances shortlisted under ToR 1 (Annex A) the following steps (A to C) were followed to select the azole substances for further assessment in this section of the report (Annex E) (Figure 4).

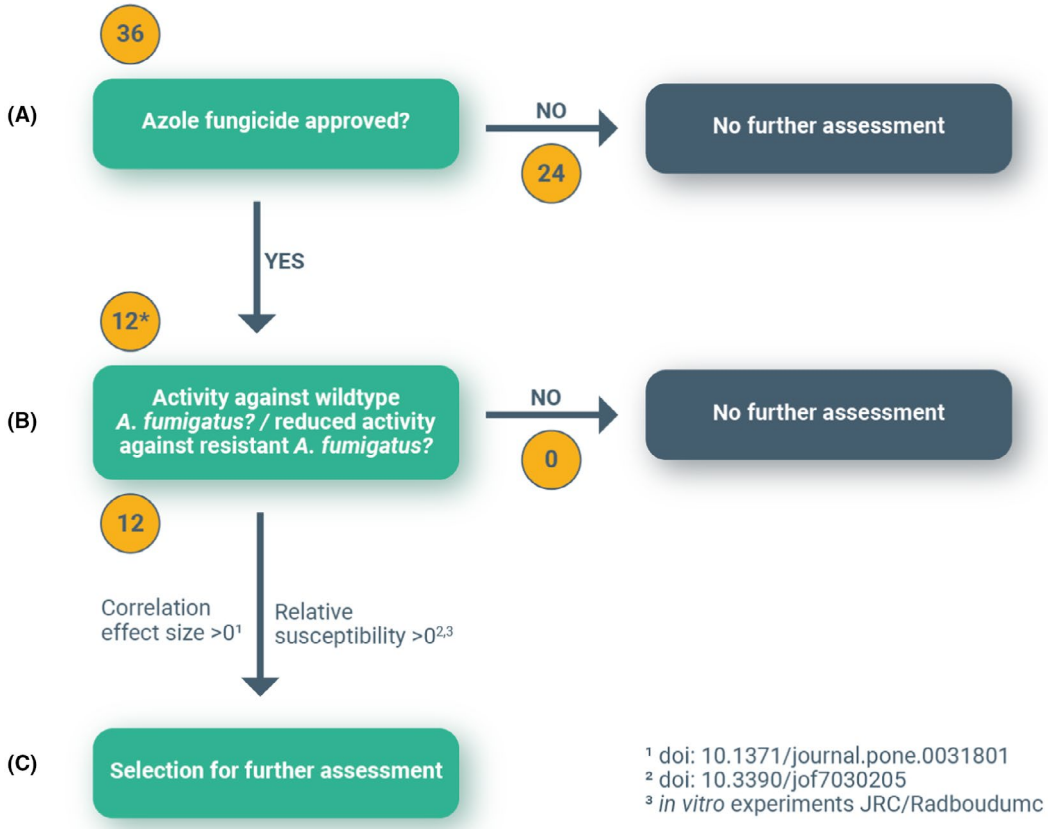

FIGURE 4 Flow chart showing the selection steps that were followed to select azole fungicides for assessment in this report. Numbers represent the number of substances selected at each step. *The active substance cyproconazole is being phased out, with only minor quantities reported in accordance with Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products (OJ L 167, 27.6.2012, p. 1–123). As a result, although being approved, no further assessment of this substance was considered necessary. Additionally, although cyazofamid is an approved active substance, it was excluded from further assessment being an outlier (cyano-imidazole, C4: complex III, as indicated in Annex A). For these reasons both substances were not further assessed.

As a first step (A), azole fungicides approved in Europe in December 2023 were considered further based on their approval status. Consequently, 12 substances were considered for further assessment, 2 substances (cyproconazole and cyazofamid) were excluded due to being phased out or being an outlier. To be able to select for resistance, an essential condition is that the azole fungicides show activity against wild-type *A. fumigatus* (Figure 4, step B). Activity of azole fungicides can be determined using a Minimum Inhibitory Concentration (MIC) test, with two reference methods available: EUCAST and CLSI reference methods. If an azole fungicide shows no activity against wild-type *A. fumigatus*, the fungicide will not select for resistance and therefore was not further considered in the assessment. The risk of selection of resistance occurs when an azole fungicide is less active against ARAf compared with wild-type *A. fumigatus*, as the growth of the wild-type isolates will be reduced at a certain concentration, but not that of the ARAf isolates causing the ARAf isolates

to become enriched. Any difference in activity of azole fungicides against wild-type *A. fumigatus* and ARAf was considered relevant for the assessment. Azole fungicides meeting these criteria (steps A and B) were selected for further assessment (Figure 4, step C). The data required to select the azole fungicides were retrieved from two publications (Jorgensen et al., 2021; Snelders et al., 2012).

As azole fungicides have been approved since the studies of Jorgensen and Snelders were published, published data on the activity of these new fungicides regarding activity against *A. fumigatus* is limited, or in vitro susceptibility data are conflicting, additional MIC results were provided to determine if these compounds should be added to the list of fungicides considered for further assessment (see Annex E).

Further assessment of the selected azole fungicides included the determination of the predicted no effect concentration for resistance selection ($PNEC_{res}$). $PNEC_{res}$ is defined as the concentration of a chemical below which no selective pressure in an ecosystem is expected. To account for uncertainties that are not accounted for in experimental setups, a safety factor ('Assessment factor', AF) is used as part of the risk assessment. To determine the AF, dose–response curves were constructed using EUCAST microbroth dilution methodology using a 96-well plate format (Figure 5).

Currently, there are no standards for $PNEC_{res}$ determination of azole fungicides and *Aspergillus* spp.

A MIC-based approach, introduced by Bengtsson-Palme and Larsson (2016), to produce $PNEC_{res}$ has been widely used for risk assessments of antimicrobials in the scientific literature. The MIC-based approach has been used in the assessment for deriving $PNEC_{res}$ for azole fungicides in *A. fumigatus*.

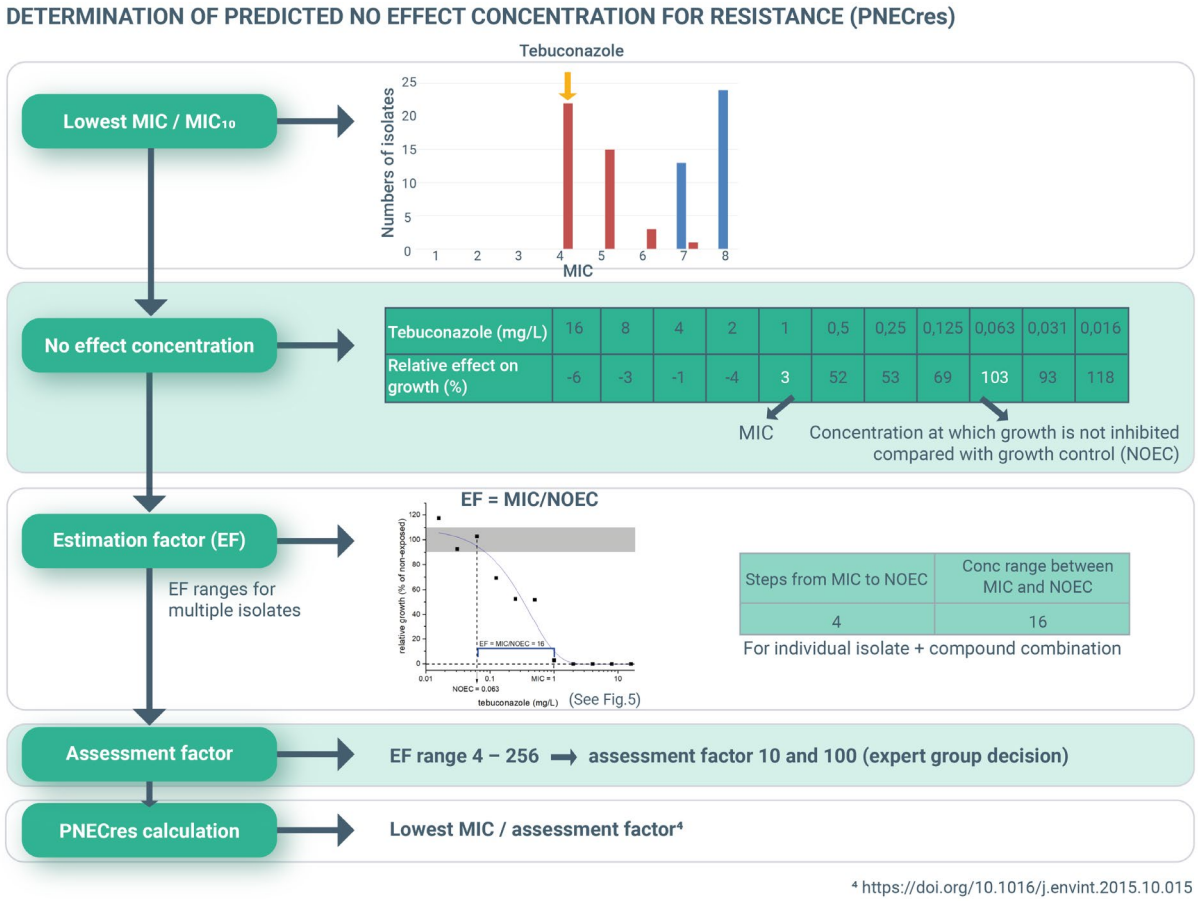

FIGURE 5 Flow chart showing the approach used to determine the predicted no effect concentrations for resistance selection ($PNEC_{res}$) in *A. fumigatus*.

In the agricultural context, a methodology has been developed that aims to identify the drivers behind environment-mediated azole resistance selection and to indicate the contribution of agricultural uses of azoles on the overall risk of environmental resistance selection. The approach chosen covers all EU authorised uses of selected azole compounds and imported commodities. The aim was to identify those azole-treated crops, by-products, processing fractions and waste materials along the full production and consumption chain that have a high probability to foster the selection of ARAf in the environment ('hotspots'). It involved a stepwise exclusion of those fractions with a low potential for hotspot formation. By examining the whole product chain, regulatory interventions may be recommended at different positions, starting at pesticide use and ending at waste treatment.

The decision scheme for the agronomic setting and the downstream processing phase consequently includes or excludes azole-treated crops and their commodities from representing a hotspot after considering the following factors: (i) azole residue data, (ii) the substrate characteristics of raw and processed commodities and (iii) environmental factors (see Annex E).

For plant protection products, the relevant scenarios deemed as high-risk for hotspot development include:

- Uses of azoles in indoor grown fruiting vegetables (e.g. tomatoes), where the plants may contain significant azole residue levels which may pose a risk for azole resistance selection at later disposal of the green waste.
- Uses of azoles in maize or sugar beet, which may lead to residues > $PNEC_{res}$ in foliage, and which, when used as silage or discarded at the field, may support the selection of resistant *Aspergillus* spp.
- Uses of azoles in wine grape production, which may lead to residues > $PNEC_{res}$ in wet pomace and which may contribute to the selection of resistant strains at disposal or by use as soil amendment or fertiliser.
- Uses of azoles in olive production, which may lead to residues > $PNEC_{res}$ in the olive wet pomace, and which may contribute to the selection of resistant strains at disposal or by use as soil amendment or fertiliser
- Uses of azoles in pome fruit production, which may lead to residues > $PNEC_{res}$ in the pome fruit wet pomace from juice production, and which may contribute to the selection of azole-resistant strains at disposal or by use as soil amendment or fertiliser
- Uses of azoles in citrus fruit production, which may lead to residues > $PNEC_{res}$ in peel or pomace e.g. from juice production and which may contribute to the selection of azole-resistant strains at disposal, by use as soil amendment or fertiliser, or at silaging.
- Crop waste in no-tillage systems and field heaps of harvest remainders (e.g. flower bulb waste), which combine environmental conditions suitable for the growth of *Aspergillus* spp. with significant azole concentrations.

Based on EU-authorised use patterns, these scenarios are characterised by the hazard characteristics of the azole fungicides in terms of activity against the wild-type *Aspergillus* spp. compared to resistant strains, substrate characteristics and residue levels, and environmental conditions that promote the growth of the fungus. Additionally, the mentioned crops are major agricultural commodities in Europe, and the by-products from their processing can accumulate in large quantities, further increasing the risk of resistance selection.

On the other side, certain scenarios like immediate consumption of treated commodities or treatment of crops with known unfavourable conditions for *Aspergillus* spp. growth (such as hops), are not considered of risk for hotspot development.

For biocidal azole applications, products for temporary preservation of freshly cut wood have been identified to have the potential for hotspot formation because freshly cut wood allows the growth of *Aspergillus* spp. and azole concentrations in treated wood are above the $PNEC_{res}$ and below the MIC of ARAf for most analysed products on the EU market.

There are several areas where further research is needed, including understanding the environmental conditions that support the growth of *Aspergillus* spp. on azole-treated wood, assessing human exposure to resistant strains, regional waste practices and the impact of combined azole use. There is also a need for more comprehensive data on the use and quantities of azole-containing products.

Azole use in veterinary medicine represents a very small percentage of total azole use and is unlikely to be a significant source of selection of resistance in the environment. As such, the focus for mitigating resistance should be on other uses of azoles. The report stresses the importance of ongoing surveillance to monitor the presence of ARAf in the environment and to inform risk assessments and management strategies.

3.6 | Prevention and control options (answering to ToR 6)

This section reports the main findings and conclusions of Annex D and Annex E, in relation to the specific parts of those Annexes answering ToR 6 of the mandate. Please refer to Annex D and Annex E for more detailed and complete information.

As requested by the mandate, a series of recommendations on possible prevention and control management options were formulated.

3.6.1 | Prevention and control of the selection of resistance in the environment

Measures related to prevention and control of the selection of resistance in the environment were formulated within Annex E of the report, following to the investigation and identification of environmental hotspots for resistance development.

It is acknowledged that measures to avoid pesticide application and to keep (soil) hygiene in plant production are already integrated in Good Agricultural/Horticultural Practice; these practices might also be effective against mass growth of azole-resistant *Aspergillus* spp., and their consideration in professional agriculture and horticulture as well as their further development is one important measure to prevent selection and dispersal of environmental resistance.

Following identification of environmental hotspots, measures are recommended to prevent the selection of azole-resistant strains in the environment, including controlled storage of organic waste, proper waste management, and responsible use and disposal of azole-treated products.

In relation to the use of azoles as PPPs, it is recommended collecting information from food industry on valorisation and disposal strategies for waste and processing by-products, and conducting experimental work to confirm or reject the hypothesised formations of hotspots. Laboratory experiments complemented by field experiments are recommended to investigate the hotspot potential of greenhouse plant waste and major food industry waste fractions for disposal under

simulation of their typical storage conditions. Field data should be collected to quantify the relevance and impact of silage from azole-treated maize and/or sugar beet on the selection and dispersal of resistant *Aspergillus* spp. Further measures include the research-based further development and propagation of Good Agricultural Practices for storage and use of field waste and food processing by-products as fertiliser or soil amendment, and the controlled storage of organic waste at communal level.

Once the technically avoidable part of azole mediated resistance selection has been addressed by Good Agricultural Practices and Good Waste Management Practices, a suitable study protocol taking into account good practices and respective regulatory data requirements for the endpoint of resistance selection or resistance development is recommended.

In relation to use of azoles as BPs, the assessment suggests that growth of ARAf on treated wood could be avoided by considering the critical azole concentration in the products or optimising application conditions to achieve a retention concentration higher than the MIC for ARAf.

Wood waste contaminated with azoles could lead to the selection of ARAf if improperly handled, such as mixing with compost. Proper waste management, including separate collection and incineration of treated wood, is recommended to minimise this risk.

Overall, it is also suggested that manufacturers and regulatory agencies perform in-depth risk assessments and inform users about the risks of resistance selection. A coordinated effort among various stakeholders, including farmers, manufactures, industrial users, waste managers, regulatory bodies and scientists, is essential to effectively address the challenge of azole resistance in *A. fumigatus*.

3.6.2 | Prevention and control of the spread of ARAf into patients

Measures related to prevention and control of the spread of ARAf into patients were formulated within Annex D of the report, following to the discussion and assessment of risks posed to humans.

Strengthening diagnostic capacity and implementing disease-specific surveillance are deemed the most crucial approaches to prevent the selection and dissemination of ARAf in the human domain.

Raising awareness about the selection and dissemination of ARAf in community settings is needed.

Experts highlighted regulation of the use of azole substances in the environment and in agriculture as well as avoiding the use of new antifungals in the environmental domain and reserving them for human medicine (or performing prior risk assessment) as options to prevent selection of azole-resistant *A. fumigatus*.

3.7 | Studies by applicants (answering to ToR 7)

This section reports the main findings and conclusions of Annex F, answering ToR 7 of the mandate. Please refer to Annex F for more detailed and complete information.

Annex F addressed three primary scientific questions (see Section 1.2.7) to guide applicants in submitting relevant data for azole product approval, focusing on the risk of cross-resistance to medical azoles.

The first question addresses the types of studies that could explore how azole fungicides may induce or select resistance. Initially, information on the azole compound, including its chemical structure and mechanism of action (fungistatic or fungicidal), is essential. In vitro studies, such as MIC tests, are critical for determining azole activity against both wild-type and resistant *A. fumigatus* strains. These tests can identify susceptibility patterns and relative MIC values across strains, which can indicate the potential for the resistant population to become dominant.

Laboratory and field studies also assess the effects of sub-MIC concentrations of azoles on resistance, exploring no-effect concentrations (NOEC) and $PNEC_{res}$. Cross-resistance, where exposure to one azole may lead to resistance against other azoles, is also investigated through interconnected studies. Additionally, studies assess whether wild-type *Aspergillus* species can survive in azole-rich environments, potentially leading to resistance through stress response mechanisms.

To address the second question, in relation to studies and data to assess the risk of resistance selection based on conditions of use, studies and data explore the risk of azole fungicides creating environmental 'hotspots' that favour resistant *Aspergillus* spp. These hotspots are defined by the coexistence of azole residues and environmental conditions that support *Aspergillus* growth. The risk assessment hinges on both hazard characteristics (azole activity and resistance selection potential) and exposure data (azole concentrations in application environments). For example, PPPs and BPs are assessed for their residue levels and environmental conditions conducive to *Aspergillus* growth.

Exposures vary across applications, such as residue levels on crop surfaces during the application period. Additionally, environments like soils, which might play a secondary role as reservoirs for resistant strains, require specific consideration. Notably, soil, although generally less supportive of resistance selection (coldspot), may act as a dispersal reservoir for resistant strains under certain conditions, such as in decaying plant matter.

Currently, no specific guidelines exist in regulatory frameworks for assessing resistance to medical azoles in non-target organisms like *A. fumigatus*. For the evaluation of such studies within the approval procedure, a preliminary framework for risk assessment was developed, which consists of a tiered approach taking into account the outcome of the assessments performed in answer to all the other ToRs of this mandate:

- Within Tier 1, MIC testing of the azole fungicide against *A. fumigatus* (and possibly other *Aspergillus* species) will indicate if the risk for resistance selection is absent or very low, if there is no activity against wild-type *A. fumigatus* isolates.
- Within Tier 2, the activity of the azole fungicide is determined against wildtype and ARAf to calculate the relative susceptibility, and further determine the possible level of risk for resistance selection.
- Within Tier 3, $PNEC_{res}$ is determined and correlated with the exposure data, such as azole fungicide maximum residue levels or predicted concentration in soil/results from environmental monitoring to evaluate the risk.

The methods presented in Tier 3 (e.g. $PNEC_{res}$), while insightful, carry a higher uncertainty and have not been validated. Continued refinement of these methods may enhance their reliability, ultimately providing more comprehensive risk assessments. Identified data gaps and recommendations for future actions are summarised to guide ongoing risk mitigation efforts.

Key recommendations include conducting in vitro susceptibility testing using validated methods to detect resistance potential and developing specific technical guidance to enhance study specifications in approval procedures. At Tier 3, any azole fungicide showing a clear risk of resistance selection may warrant environmental monitoring.

3.8 | JRC experimental studies

This section reports the main findings and conclusions of Annex G, reporting a set of experimental studies conducted by JRC during the course of the mandate. Please refer to Annex G for more detailed and complete information.

The aim of the JRC's experimental study was to investigate the susceptibility of *A. fumigatus* exposed to azole and non-azole compounds. Clinical, soil and compost material samples were used in the study to provide valuable insights on how these substances influence the incidence of antifungal resistance.

Data from in vitro studies (MIC tests) were combined with sequencing results of the *cyp51* gene to identify possible mutations causing the resistance. The 16 substances (amisulbrom, climbazole, clotrimazole, fenbuconazole, fluconazole, imazalil, ipconazole, metconazole, miconazole, penconazole, pyrifenox, pyrisoxazole, prochloraz, tebuconazole, tetraconazole, triforine) used in the MIC tests generally displayed a similar pattern activity against all the wild-type *A. fumigatus* isolates. Among the non-azole substances, only pyrisoxazole (pyridine) was active against *A. fumigatus*, while tetraconazole and amisulbrom, despite being triazoles, did not show any activity against the fungus. When looking at the mutant strains, a generally similar pattern activity was observed against *A. fumigatus* isolated from soil, compost material and clinical strains, possibly due to the presence of the same mutation across the isolates. The implications of these findings may provide deeper insights into the relationship between *A. fumigatus* and its ability to develop resistance to azole compounds and other antifungal agents.

3.9 | Data gaps and recommended actions to address data gaps/uncertainties (answering to ToR 8)

For each ToR 1–7, respective uncertainties and data gaps were identified. EFSA, ECDC, ECHA, EEA and EMA, with the scientific support from JRC, formulated a series of recommendations to be considered by regulators (European Commission and Member States), researchers and relevant industry sectors as priority areas to be supported at EU level within the field of monitoring, reporting, research and epidemiological investigation on this topic. The recommendations would allow addressing such gaps and reducing them in the future. Table 1 reports a summary of the main data gaps, and respective recommended actions to address them, identified for every ToR within Annexes A–G, and refers to the relevant Annex where more detailed information and recommendations can be found. Each of those Annexes include a dedicated section with a table reporting detailed information.

TABLE 1 Data gaps and recommended actions to address data gaps/uncertainties (e.g. research needs, data collections etc.) with respect to the different ToRs and relevant Annex where more detailed information can be found.

Topic area	Data gaps	Recommended actions	Relevant annex
Use of azole fungicides	• Lack of the appropriate level of detail of data on the type of use of specific products/substances in all the regulatory frameworks considered (PPP, BPR, VMP, REACH)	• Establishment of a mandatory reporting system at national level with the appropriate level of detail related to the substance/product used and its specific application (e.g. crop of application) • Overcoming confidentiality issues that limit dissemination and analysis of the data collected and possible double reporting of data within different regulatory frameworks	A

TABLE 1 (Continued)

Topic area	Data gaps	Recommended actions	Relevant annex
Epidemiology of ARAf (patient-related data)	• Incomplete information on patients' prior exposure to azole therapy • Lack of information on epidemiological investigations of ARAf-infected patients; most surveillance involves *Aspergillus* pathogen surveillance, without taking into account *Aspergillus* diseases • Lack of studies evaluating the impact of different antifungal treatment strategies on the outcome of patients with IA due to azole-resistant *Aspergillus* spp.	• Improving data collection, analysis, reporting and epidemiological investigations from ARAf-infected patients • Develop approaches to classify patients with *Aspergillus* diseases for surveillance purposes to monitor the clinical impact of surveillance • Performing randomised clinical studies to evaluate the impact of antifungal treatment strategies	B/C
Epidemiology of ARAf (prevalence data)	• Lack of data on prevalence of azole resistance in *Aspergillus* spp. in the different domains (humans, environment, animals) • Lack of data on ARAf-related contamination in different specific environments (domestic environment, hospital environment, animal and plant farming environment, etc.) • Lack of studies on the relative public health relevance posed by the different domains	• Design and implementation of standardised prevalence studies, at national and EU level, with appropriate sampling sizes and number of isolates collected within the different domains • Strengthen genomic testing for the identification of ARAf strains involved and specific azole resistance mechanisms • Screening of environments, including working environments in all domains, related personnel, domestic/non-domestic environments close to patients, farm/pet animals and their environment, including comparison with ARAf clinical isolates	C
Spread of ARAf	• Lack of studies making use of genotyping data to investigate clonal spread of ARAf strains	• Design and implementation of molecular epidemiology studies aimed at defining the directionality of spread of azole resistance and of ARAf strains • Investigation of pathways for cross-border dissemination of ARAf strains • Investigation of pathways for transfer of ARAf from industrial to residential environments (e.g. through flower bulbs, compost, etc.)	B/C
ARAf monitoring and surveillance	• Absence of ARAf monitoring and surveillance system	• Establishment of harmonised EU monitoring and surveillance system, including genomic surveillance, within the human, animal and environmental domains (aquatic, terrestrial, air)	C
Diagnostic methods	• Lack of standardisation in the methodologies used for characterisation of ARAf strains • Lack of routine performance of antifungal susceptibility testing • Limited mycology reference centres	• Standardisation of (environmental) sampling and typing techniques • Development of guidelines to define and increase routine use of antifungal susceptibility testing • Establishment of national reference laboratories (NRLs) for mycology and of a related EU Network of NRLs • Development of commercially available tests	B/C
Hazard-related data	• Lack of data on certain aspects of resistance development in *Aspergillus* spp. other than *Aspergillus fumigatus* • Lack of knowledge on the combined effect of exposure to different azoles in the selection of azole resistance in *A. fumigatus*	• Investigation of additional underlying mechanisms of azole resistance in *Aspergillus* spp. from the different domains, including animals • Investigation of azole susceptibility in *Aspergillus* spp. other than *A. fumigatus* • Investigation of chemical structures other than azoles that may favour relevant mutations in *A. fumigatus* • Investigation on effect on resistance development of substances other than triazoles and imidazoles with similar mode of action (G: Sterol biosynthesis in membranes, G1: C14 demethylase in sterol biosynthesis erg11/cyp51; e.g. pyrisoxazole) (see Annex G) • Research in combined exposure of *A. fumigatus* to different azoles	C/E/G

(Continues)

TABLE 1 (Continued)

Topic area	Data gaps	Recommended actions	Relevant annex
Residues of azole fungicides in the environment	• Environmental persistence of azole substances • Lack of data on true residue levels in the crop waste and wood waste to be used for risk assessment purposes • Lack of data on extent of human exposure to azoles in the environment • Lack of appropriate monitoring of azole substances in ground/surface waters • Lack of appropriate monitoring of azole substances in terrestrial environment (e.g. soil)	• Monitoring of azoles approved in the past but not EU-approved anymore in the environment to investigate environmental persistence • Monitoring of residues levels in crop waste and wood waste fractions • Investigation of human exposure levels to azoles in certain environments (e.g. from treated wood, etc.) • Enhancing monitoring and reporting of the presence of azole substances in ground/surface waters and in terrestrial environment (e.g. soil)	A/E
Hotspots	• Lack of data on conditions favourable to growth of *A. fumigatus* in certain substrates • Lack of data on waste management practices for certain substrates • Lack of data on hotspot potential represented by certain substrates • Lack of data leading to the use of assumptions in risk assessment methodology used to identify environmental hotspots (e.g. estimation of assessment factors, calculation of $PNEC_{res}$, effect of azole exposure in laboratory vs. field conditions, etc.)	• Investigation of data on conditions favourable to growth conditions of *A. fumigatus* in certain substrates (e.g. wood, agricultural no-tillage systems, etc.) • Collection of data on waste management practices in certain substrates (e.g. industrial food processing, household waste, green waste, construction and demolition waste) • Design and implementation of higher tier experiments in more realistic environmental conditions in certain substrates (e.g. industrial food processing waste, greenhouse plant waste, processed feed, etc.) • Design and implementation of field studies to validate assumptions used within the risk assessment methodology used to identify environmental hotspots	E

4 | CONCLUSIONS

In relation to the use of azole fungicides:

- Azoles represent the main class of substances for prevention and treatment of *Aspergillus* diseases. They are also widely used in the EU as plant protection products (PPP) and also used in biocides (BPs), industrial chemicals and veterinary medicinal products (VMPs).
- The main chemical classes of azoles studied in this report and deemed relevant/responsible for selection of resistance are triazoles and imidazoles.
- Data collected from the competent authorities of EU/EEA Member States that were able to provide data through a survey, although incomplete, show that, overall, around 120,000 tonnes of azoles were reported to be sold on the EU/EEA market between 2010 and 2021 for uses other than as human medicines.
- Most of the azoles are used as PPPs (more than 119,000 tonnes), with a stable trend, on average 10,000 tonnes a year, with alterations in the different countries.
- During the time period analysed, it was not possible to draw any firm conclusions regarding geographical trends in sales and use of azoles as PPPs, BPs, VMPs and industrial chemicals, as most of the substances were used throughout EU/EEA.
- A rough comparison made for year 2021 showed that the amount of triazole and tetrazole derivatives for systemic use consumed by humans (including both the community and hospital sectors) was about 1,000 times smaller than the amount of azole substances reported for use as PPPs, BPs, VMPs and industrial chemicals.

In relation to the link between the environmental use of azole fungicides and the selection of resistance to medical azoles, the epidemiology of human infections with azole-resistant *Aspergillus* spp. and the impact for human health:

- There is substantial evidence that supports a link between azole resistance selection through azole fungicide exposure in the environment and cross resistance selection to medical azoles in *Aspergillus* species (environmental route of resistance selection).
- This link has been primarily shown for *A. fumigatus* and remains less clear for other *Aspergillus* species.
- This link has been primarily shown for PPPs and is less clear for BPs and industrial chemicals and seems unlikely for VMPs.
- The most frequently identified molecular resistance mechanism in azole-resistant *A. fumigatus* (ARAf) isolates from azole-naïve patients was the TR_{34}/L98H mutation, followed by the TR_{46}/Y121F/T289A mutation, as well as the G54E and Y121F mutations.

- Numerous studies have documented the clonal dissemination of strains with the above types of molecular resistance mechanisms across clinical, environmental and agricultural settings. The rapid global spread of clonal lineages of ARAf with TR_{34} and TR_{46} mutations (often in combination with other genetic alterations), as well as the risk of spread related to the importation and exportation of agricultural products from hotspots for azole-resistant *Aspergillus* spp., are of concern.
- ARAf has been reported in multiple EU Member States, although international resistance surveillance programmes are currently lacking.
- *A. fumigatus* is the primary cause of *Aspergillus* diseases in the EU including invasive aspergillosis (IA), chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA).
- While azole resistance in *A. fumigatus* infections is high in Europe, varying rates have been reported in other parts of the world. The reported prevalence of ARAf in human *A. fumigatus* infections varied among the different presentations of the disease and between geographic regions: 0.7%–63.6% (IA), 5.9%–59.2% (CPA), 2.3%–42.8% (ABPA). This variation can be attributed to various factors such as differences in antifungal exposure, environmental factors and the emergence of specific azole-resistant strains, but also due to detection bias in certain areas.
- Risk factors associated with aspergillosis due to ARAf, and more generally azole-resistant *Aspergillus* spp., include underlying immunosuppression, such as patients with hematologic malignancies or solid organ transplantation, chronic obstructive pulmonary disease, cystic fibrosis, chronic granulomatous disease and immunosuppressive therapy, as well as long-term hospitalisation (especially in areas with a high prevalence of azole resistance in *Aspergillus* spp.). Exposure to agricultural sites, woodwork and ground maintenance can potentially increase the risk for the acquisition of azole-resistant *Aspergillus* spp.
- Clinical implications of azole resistance have been reported in IA (36–100% mortality rates) but are less well documented in CPA and ABPA.
- Patient-to-environment transmission of ARAf is considered rare, as *Aspergillus* spp. primarily spread through the release of conidia.
- Overall:
  - Available evidence supports the hypothesis that transmission of ARAf occurs from the environment to humans.
  - Azole usage outside the human domain is likely or very likely to contribute to the selection of ARAf isolates that could cause severe disease such as IA, but the extent of this contribution needs to be better understood.

In relation to environmental hotspots for selection of azole resistance in *Aspergillus fumigatus*:

- Ecological selection dynamics for ARAf include activity of azole fungicides against *A. fumigatus*, substrates that supports the growth of *A. fumigatus* and the presence of fungicide residues that exceed the predicted no effect concentration for resistance selection ($PNEC_{res}$).
- These dynamics support azole resistance selection in specific scenarios referred to as environmental hotspot for resistance selection.
- For PPPs, the relevant scenarios identified include stockpiling of agricultural waste and their possible use as soil amendment or fertiliser for several agricultural crops such as indoor growing fruiting vegetables, wine grape, maize, sugar beet, olives, pome fruit, citrus fruit and field heaps.
- For BPs, the relevant scenarios identified include freshly cut wood.
- For VMPs and industrial chemicals, no relevant scenarios are identified since azole use in these fields represents a very small percentage of total azole use and is unlikely to be a significant source of selection of resistance in the environment, and industrial chemicals are mostly used as intermediates to manufacture other substances including PPPs, BPs or VMPs.

5 | RECOMMENDATIONS

As an outcome of this report EFSA, ECDC, ECHA, EEA and EMA, with the scientific support from JRC, formulated a series of recommendations for consideration by regulators and by the relevant industry sectors.

In relation to prevention and control of the selection of azole resistance in *A. fumigatus* in the environment and of its further spread to patients, it is recommended to:

- consider carefully the need of an azole substance being used in a PPP or BP;
- implement and further develop Good Agricultural/Horticultural Practices in professional agriculture and horticulture;
- ensure controlled storage of organic waste, proper waste management and responsible use and disposal of azole-treated products;
- collect information from food industry on valorisation and disposal strategies for waste and processing by-products;
- carry out experimental work to confirm or reject the hypothesised formation of hotspots, including both laboratory and field experiments;
- further develop and propagate Good Agricultural Practices for storage and use of field waste and food processing by-products as fertiliser or soil amendment and the controlled storage of organic waste at communal level;

- consider critical azole concentration in treated wood or optimise application conditions to achieve a retention concentration higher than the minimum inhibitory concentration (MIC) for ARAf;
- implement proper wood waste management, including separate collection and incineration of treated wood;
- strengthen diagnostic capacity, implement disease-specific surveillance, and raise awareness about the selection and dissemination of ARAf in community settings;
- support research and development of both new fungicides with novel mechanism of action that do not have cross-resistance with antifungals used in human medicine, and new antifungal medicinal products active against azole-resistant *Aspergillus* spp.;
- provide or perform a prior assessment of the risks for cross-resistance with antifungals used in human medicine before approving a new fungicide or renewing an existing approval;
- consider including specific requirements related to the public health risks of antifungal resistance within regulatory requirements related to approval of new fungicides or renewal of existing approvals.

In relation to recommended studies or information that could be provided by applicants when submitting applications for the approval of azole substances for the different uses, other than as human medicines, currently regulated under the EU legislative framework:

- Topics to be covered by the studies or information that could be provided by applicants when submitting applications for the approval of azole substances for uses other than as human medicines are listed. Among them, performance of in vitro susceptibility testing based on validated and standardised methods can already be recommended to flag active substances that may potentially contribute to cross-resistance.
- Based on the study topics identified, it is recommended that further specific guidance is developed to provide technical specifications for specific studies to be submitted within approval procedures.
- For the evaluation of such studies within the approval procedure, a preliminary framework for risk assessment was developed, which consists of a tiered approach taking into account the outcome of the assessments performed in answer to all the other terms of reference of this mandate.
- Once technical specifications for specific studies to be submitted with approval procedures will be compiled, it is recommended to refine such preliminary framework for risk assessment in order to allow their integration in a defined risk assessment methodology.

Several data collection and reporting activities as well as scientific studies and research were recommended to address the data gaps identified (see Section 3.9).

ABBREVIATIONS

ABPA	allergic bronchopulmonary aspergillosis
AF	assessment factor
AQ	assessment question
ARAf	azole-resistant *Aspergillus fumigatus*
BP	biocidal product
BPR	Biocidal Products Regulation
CPA	chronic pulmonary aspergillosis
ECDC	European Center for Disease Prevention and Control
ECHA	European Chemicals Agency
EEA	European Environment Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EU/EEA	European Union/European Economic Area
IA	invasive aspergillosis
JRC	European Commission's Joint Research Centre
L-AMB	liposomal amphotericin B
MIC	minimum inhibitory concentration
NOEC	no-effect concentration
NRL	National Reference Laboratory
PNEC	predicted no effect concentration
$PNEC_{res}$	predicted no effect concentration for resistance selection
PPP	plant protection product
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ToR	term of reference
VMP	veterinary medicinal product

GLOSSARY

Aspergillosis	Disease caused by *Aspergillus* species, including *A. fumigatus*, in humans, mainly involving the lung. The disease manifestation depends on the underlying host defence. These include allergic reaction to spores (allergic bronchopulmonary aspergillosis, ABPA), chronic infection of the lung (chronic pulmonary aspergillosis, CPA) or tissue invasive infections in immunocompromised patients (invasive aspergillosis, IA).
Azoles	Azoles are a class of five-membered heterocyclic compounds containing a nitrogen atom and at least one other non-carbon atom (i.e. nitrogen, sulfur or oxygen) as part of the ring. They are classified into two groups: those with two nitrogen atoms in the azole ring (the imidazoles) and those with three nitrogen atoms in the azole ring (the triazoles). Azoles have antifungal properties and work by inhibiting the cytochrome P450 dependent enzyme lanosterol 14-alpha-demethylase (also referred to as demethylase inhibitor – DMI), which converts lanosterol to ergosterol, the main sterol in the fungal cell membrane. Depletion of ergosterol damages the cell membrane resulting in cell death. Azole compounds are used as active ingredients in medicines and pesticides for treatment of fungal disease in humans and animals, crop protection, as biocides and for other uses as described in the current report and its Annex A.
Biocides or biocidal products	Biocides (or biocidal products) are used to protect people and animals against harmful organisms, like pests or bacteria. Each biocidal product contains one or several active substances that are designed to control viruses, fungi and other microbes before they cause harm. In some cases, biocides are designed to repel or attract organisms, such as insects. For more information, see also ECHA website (https://www.echa.europa.eu/hot-topics/biocides). A EU legal definition is provided in Regulation (EC) 528/2012).
Azole biocides	Biocidal products consisting of an active substance of the azole chemical class. They are intended to destroy, deter, render harmless or exert a controlling effect on harmful fungi. They are mostly used to preserve materials such as wood.
Plant protection products	Products used to protect, preserve or influence the growth of desirable plants or to destroy or control the growth of unwanted plants or parts of plants. A EU legal definition is provided in Regulation (EC) 1107/2009).
Fungicides	Fungicides are pesticides (both chemical compounds and biological organisms) that kill or prevent the growth of fungi and their spores. They can be used to control fungal plant diseases that damage plants, including rusts, mildews and blights, or to control the growth of mould and mildew in other settings (e.g. industrial settings).
Azole fungicides	Fungicides consisting of an active substance from the azole chemical class.
Antifungals	Substances used to treat or prevent human/animal fungal infections.
Medical azoles (or azole medicinal products)	Antifungal agents of the azole class. Medical azoles with activity against most *Aspergillus* spp. including *A. fumigatus* include itraconazole, voriconazole, posaconazole and isavuconazole. The medical azoles can be administered intravenously or orally. Medical azoles are also used in veterinary medicine.
Industrial chemicals	Chemical substances manufactured or imported in/into the EU in quantities of one or more tonnes/year and registered under REACH Regulation to the European Chemicals Agency. For more information, see ECHA website: https://echa.europa.eu/regulations/reach/understanding-reach
Veterinary medicinal product	Medicinal substances or combinations of substances used to treat, prevent or diagnose disease in animals. A EU legal definition is provided in Art. 4 (1) of Regulation (U) 2019/6.
Azole resistance in *Aspergillus* spp.	Ability of *Aspergillus* species (e.g. *A. fumigatus*) to withstand the effects of a harmful chemical compound of the azole class. The effects of resistance are treatment failure in the clinic and yield loss in agriculture.
Azole cross-resistance *Aspergillus* spp.	Resistance mutations that develop in *Aspergillus* species (e.g. *A. fumigatus*) through exposure to azole fungicides, that reduce the activity not only of azole fungicides but also that of one or more chemically related medical azoles.

Azole-naïve patient	Patient without a history of previous azole exposure, either for prophylaxis or treatment.
Human domain/source	Refers to *Aspergillus* spp. isolates originating from human patients.
Animal domain/source	Refers to *Aspergillus* spp. isolates originating from companion and/or food-producing animals.
Agricultural domain/source	Refers to *Aspergillus* spp. isolates originating from the following environments: agriculture fields, crops and plant production settings, flower beds, seeds, fruits, vegetables and plant bulbs.
Environmental domain/source	Refers to *Aspergillus* spp. isolates originating from the following environments: wood chips, dust, wastewater, groundwater, sewage, surface water, compost pile, soil, waste treatment plant, air, public gardens and hospitals.
Hotspot	Environment that supports the selection and dispersal of azole-resistant *A. fumigatus*.
Coldspot	Environment in which development or selection of azole-resistant *A. fumigatus* is not supported and resistance levels do not exceed that of the background level.
Predicted no effect concentration (PNEC)	Concentration of a substance below which an unacceptable effect most likely will not occur, widely used for risk assessment and in environmental policy and regulation.
Predicted no effect concentrations for resistance selection ($PNEC_{res}$)	Application of this concept has been extended to fungi within this report. The concentration at which an antibiotic/antifungal/fungicide does not have the ability to select for antibiotic/antifungal/fungicide resistant strains. The $PNEC_{res}$ is proposed to be based on experimental data that determine the no effect concentration (NOEC) of an antibiotic/antifungal/fungicide against a specific microorganism. The NOEC is below the minimal inhibitory concentration (sub-MICs) of susceptible microorganisms and predicts the lowest concentration at which, theoretically, the selection of a resistant strain would begin to occur. To generate $PNEC_{res}$ it is proposed to apply a safety factor (assessment factor, AF) to account for remaining uncertainties, not covered in the experimental setup. Practically, the ratio between MIC/NOEC provides a measured estimation factor (EF) to derive generic AFs that can be used to calculate compound specific $PNEC_{res}$ from their lowest MICs determined from susceptible strains.
Susceptibility	Also referred to as sensitivity, implies that the cultured fungal pathogen growth will be inhibited by exposure to specific antifungals.
Antifungal MIC	Lowest concentration, recorded in mg/L, of an agent that inhibits the growth of a fungus in vitro using a standardised method.
MIC_{10}	MIC value (compound concentration) able to inhibit the growth of 10% of a population of isolates tested.
MIC_{50}	MIC value (compound concentration) able to inhibit the growth of 50% of a population of isolates tested.
Relative susceptibility	The activity of a compound against a population of microorganisms in comparison with another population. The relative susceptibility is often determined as the number of twofold dilution step differences in concentration of MIC_{50}'s of the two groups (log_2MIC_{50} of population (A)/log_2MIC_{50} of population (B)). A log_2 MIC difference of ≥ 3 is commonly considered significant.

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ANNEX A

Use of azole fungicides (answering ToR 1)

Annex A is available at the following link: https://doi.org/10.5281/zenodo.14221845

ANNEX B

Azole resistance mechanisms, and link between the use of azole fungicides and azole-resistant Aspergillus spp. infections in humans (answering ToR 2)

Annex B is available at the following link: https://doi.org/10.5281/zenodo.14223358

ANNEX C

Epidemiology of human infections (answering ToR 3)

Annex C is available at the following link: https://doi.org/10.5281/zenodo.14223384

ANNEX D

Risk assessment for human health (answering ToR 4 and part of ToR 6)

Annex D is available at the following link: https://doi.org/10.5281/zenodo.14223402

ANNEX E

Environmental hotspots and Prevention and control options (answering ToR 5 and part of ToR 6)

Annex E is available at the following link: https://doi.org/10.5281/zenodo.14223436

ANNEX F

Studies by applicants (answering ToR 7)

Annex F is available at the following link: https://doi.org/10.5281/zenodo.14223460

ANNEX G

Experimental studies performed by JRC

Annex G is available at the following link: https://doi.org/10.5281/zenodo.14223472