European Commission

# Azole Fungicides proposed for the 3rd WL under the Water Framework Directive



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**Use of azole fungicides**

Azoles represent the most common class of fungicides[1]. They are used worldwide as therapeutic agents against pathogenic fungi (antifungal pharmaceuticals for the treatment of superficial and systemic fungal infections). These substances are not only active against human pathogenic fungi but are equally active against the plant pathogenic fungi[2]. Indeed, azoles are also used in the food and agricultural industries to prevent and treat fungal infections of crops. Plant pathogenic fungi also produce several mycotoxins, which cause serious harm to the consumers. The use of azoles as PPP is advantageous over other fungicides because they are less expensive, long-lasting and have broad-spectrum activity[2].

Azoles have also industrial applications, for example, they are added to a variety of products such as paints and coatings that prevent fungal growth. Moreover, they are also used to preserve wood[3].

The azole compounds suggested by the JRC for the next WL belong to two use categories, antifungal pharmaceuticals (e.g. clotrimazole, fluconazole, miconazole) and plant protection products (PPP) and/or biocides (e.g. epoxiconazole, ipconazole, imazalil, metconazole, penconazole, prochloraz, propiconazole, tebuconazole, tetraconazole).

Table 1 lists the azole compounds proposed by the JRC for the WL. The table includes the name of the substance, CAS number, chemical group, use and mode of action (MoA) and PNEC value.

Table 1. Azole compounds proposed by the JRC for the WL.

| Azole compound | CAS | Chemical group | USE | Mode of action | PNEC (µ/l) |
|---|---|---|---|---|---|
| Clotrimazole | 23593-75-1 | Imidazole | Pharmaceutical Superficial infections | Inhibition of **ergosterol** biosynthesis | 0.02 |
| Fluconazole | 86386-73-4 | Triazole | Pharmaceutical Systemic and superficial infections | Inhibition of **ergosterol** biosynthesis | 0.25 |
| Miconazole | 22916-47-8 | Imidazole | Pharmaceutical Superficial infections | Inhibition of **ergosterol** biosynthesis | 0.2 |
| Epoxiconazole | 133855-98-8<br>135319-73-2 | Triazole | PPP | Inhibition of **ergosterol** biosynthesis | 0.18 |
| Imazalil | 35554-44-0 | Imidazole | PPP | Inhibition of **ergosterol** biosynthesis | 0.8 |
| Ipconazole | 125225-28-7 | Triazole | PPP | Inhibition of **ergosterol** biosynthesis | 0.044 |

| Azole compound | CAS | Chemical group | USE | Mode of action | PNEC (µ/l) |
|---|---|---|---|---|---|
| Metconazole | 125116-23-6 | Triazole | PPP | Inhibition of **ergosterol** biosynthesis | 0.029 |
| Penconazole | 66246-88-6 | Triazole | PPP | Inhibition of **ergosterol** biosynthesis | 1.7 |
| Prochloraz | 67747-09-5 | Imidazole | PPP | Inhibition of **ergosterol** biosynthesis | 0.161 |
| Propiconazole | 60207-90-1 | Triazole | Biocide | Inhibition of **ergosterol** biosynthesis | 0.095 |
| Tebuconazole | 107534-96-3 | Triazole | PPP Biocide | Inhibition of **ergosterol** biosynthesis | 1 |
| Tetraconazole | 112281-77-3 | Triazole | PPP | Inhibition of **ergosterol** biosynthesis | 1.9 |

**Chemical structure**

Azoles are heterocyclic compounds structurally related to pyrroles in which one or more carbon atoms of the ring are replaced by nitrogen, oxygen or sulphur. If the ring has two nitrogen atoms, the resulting compound is an imidazole. If there are three nitrogen atoms, the compounds are called triazoles (Figure 1). The azoles proposed by the JRC are derivatives of the single-ring compounds imidazole or either the 1,2,3 or 1,2,4 isomers of triazole. The imidazole group includes clotrimazole, miconazole, imazalil (enilconazole) and prochloraz, while the triazole group comprises fluconazole, epoxiconazole, ipconazole, metconazole, penconazole, propiconazole and tebuconazole.

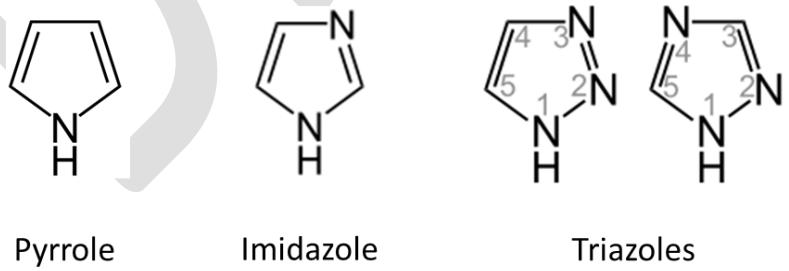


Figure 1. Chemical structure of pirroles, imidazoles and triazoles.

**Mode of action (MoA)**

The azole compounds have a common MoA, they competitively inhibit the fungal CYP51-class cytochrome P450 superfamily enzyme 14α-*sterol demethylase* in a dose-dependent manner[3]. CYP51 enzymes are essential components of the pathway leading to the synthesis of ergosterol, a major sterol of the plasma membrane of most fungi[3]. Azoles also act by inhibiting a similar functioning enzyme (24-methylene dihydrolanosterol demethylase) in the fungal cell[2] (Figure 2). Ergosterol maintains the membrane rigidity, stability and integrity. In most fungi, azoles exert a dual antimicrobial effect. Firstly, ergosterol depletion causes instability of the membrane, which leads to growth and proliferation inhibition. Secondly, fungal CYP51 inhibition causes the accumulation of different methylated metabolites, which toxic to the fungal cell[3].

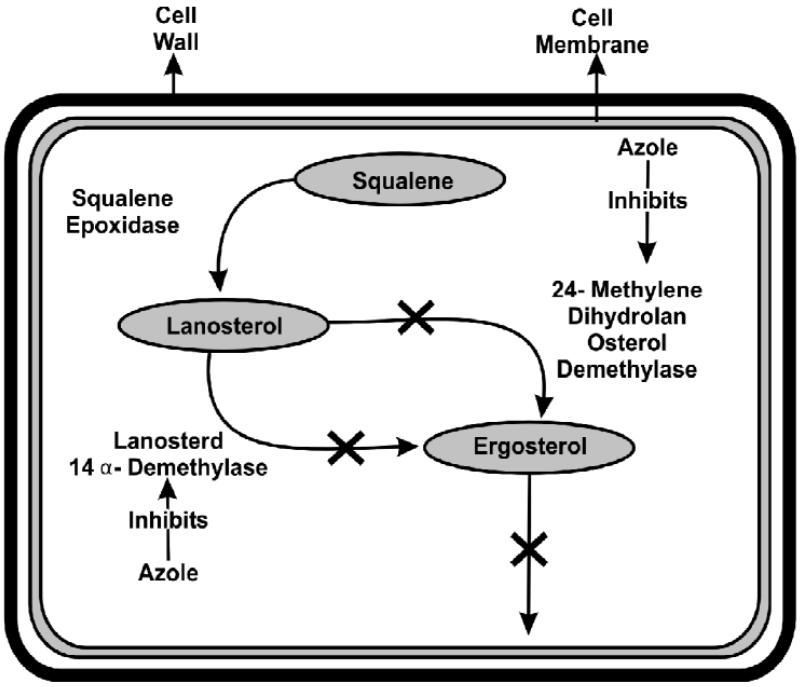


Figure 2. Biosynthesis of Ergosterol inhibited by azole compounds (adapted from Shandy et al. 2014[2])

Azoles at higher concentrations exhibit fungicidal activity while at lower concentrations exhibit fungistatic activity[2].

Besides fungi, CYP51 enzymes are essential for sterol synthesis in different eukaryotes and bacteria. Therefore, they may cause toxic effects also to other organisms including the host. Triazoles have been shown to discriminate between fungal and mammalian P450 enzymes better than the imidazoles, which makes them safer for humans to use. Nevertheless, they are not completely free of adverse effects[3]. Currently, imidazoles (e.g. miconazole, clotrimazole) are limited primarily to the treatment of superficial mycoses, while triazoles (e.g. fluconazole) are systemically administered. Azole derivatives with 1, 2, 4-triazole ring in place of imidazole

have a better pharmacokinetic profile with better fungicidal activity and lower toxicity[1]. Newer azoles are under development in an effort to combat resistant pathogens while improving upon the tolerability and ease of administration[4].

**Azole resistance**

Fungal drug resistance can be primary (intrinsic) or secondary (acquired). Secondary resistance can emerge *de novo* through a selection process when the organism is exposed to the drug for a prolonged period[3]. The mechanisms by which fungi develop resistance to a drug depend mainly on the drug's MoA and the number of target sites. The fungal cell can acquire resistance through the following mechanisms: decreasing the affinity of the drug for the target (mutations in the drug target); overexpressing the cellular target; up-regulating genes related to drug efflux; reducing the cellular drug influx rate; modifying the drug metabolic degradation or forming biofilms. Unlike antimicrobial resistance in bacteria, in fungi horizontal transfer of resistance-conferring genes has not been demonstrated[3].

**Risk assessment**

According to the European environmental agency (EEA report, 2020)[5] fungicides are seldom seen as a water quality problem. This statement is based on the comparison of exceedance rates of different pesticides (herbicides, insecticides and fungicides) in surface waters (information retrieved from the Waterbase – Water quality) in the time period 2007-2017 (Figure 3). The substances with higher exceedance rate were 14 herbicides, 17 insecticides and 6 fungicides, being insecticides the groups showing the highest values.

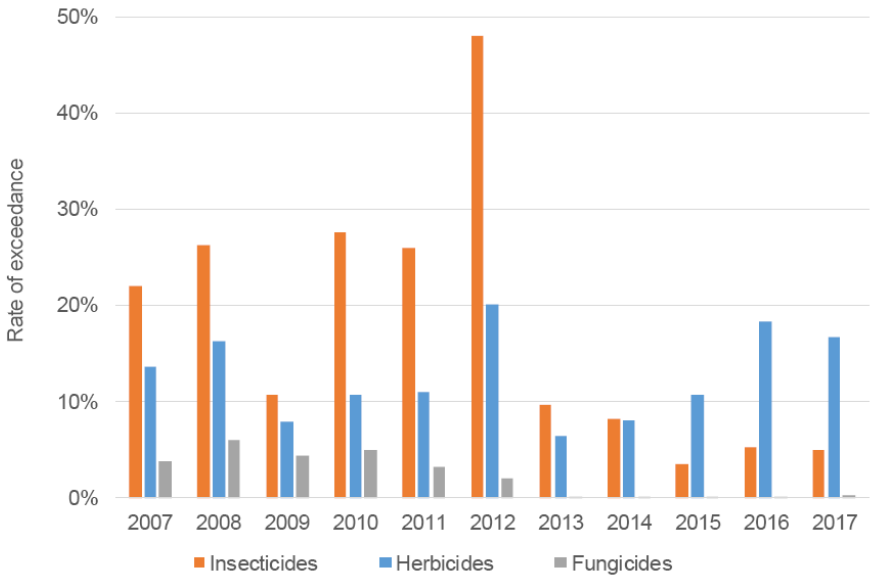


Figure 3. Rate of exceedances of the three usage groups of pesticides from 2007 to 2017 in surface waters (EEA report, 2020)[5].

For the comparison, the EEA report 2020[5] considered a total of 24 fungicides among which five were azole compounds (triazoles). Four of the azoles considered in the EEA report 2020[5] (epoxiconazole, penconazole, propiconazole and tebuconazole) are also proposed by the JRC within the group of azoles for the 3rd WL. The rest of fungicides considered belong to other chemical groups with different mode of action (MoA). Among the azoles, epoxiconazole and propiconazole showed the highest exceedance rates which in both cases were below 1 %. These substances (assessed individually) have a lower rate of exceeding samples comparing to the group of insecticides. It is important to highlight that the most toxic azole compounds such as metconazole (PNEC=0.029 µg/L) are not considered in the EEA report 2020[5].

Since azoles have a common MoA., they could be considered together for risk assessment (cumulative assessment of risk).

The cumulative assessment (CA) of risk posed from a mixture of substances with a similar MoA is estimated via summing the risk quotients of individual substances, according to the following formula:

$$RQ_{mixture} = \sum_{i=1}^{n} RQ_i \ ,$$

where $RQ_i = MEC_i/PNEC_i$ is a risk quotient of *i-th* substances in the mixture. The CA of risk concept could be applied only when the individual substances in the mixture are monitored simultaneously at each monitoring site. The above approach is exemplified as follows in a case study carried out by the JRC.

**Cumulative risk assessment of azole compounds proposed to be included in the 3rd WL**

The purpose of this case study is to show that the rate of exceedances considerably increase if the azole compounds are evaluated together applying the cumulative risk assessment approach[6,7] (summing the individual risk quotients of substances with a similar MoA).

The case study considers the 9 azole compounds proposed to be included in the 3rd WL. The sole criterion for the selection of substances participating in the case study was each substance to be monitored simultaneously in as much as possible countries and to have a considerable amount of samples. According to the available data from the prioritization exercise 4 substances (Epoxiconazole, Prochloraz, Propiconazole and Tebuconazole) fulfilled the above conditions (see Table 2 and Table 3). We found totally 13086 cumulative samples when all 4 substances were measured simultaneously in 3 MS, and these have been used in the current case study. The remaining 5 azole compounds were not included because they have either insufficient or low-quality monitoring data (Ipconazole is missing any data).

Table 2: List of MS monitored the selected azole PPP substances (Sc3 dataset). The case study is based on data from 3 MS which monitored all 4 substances.

| Substance | BG | CZ | DE | Fi | FR | IE | IT | LU | NL | SE | UK | Number of MS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Epoxiconazole | | x | x | x | x | x | | x | | x | | 7 |
| Prochloraz | | | x | x | x | | | x | x | x | x | 7 |
| Propiconazole | | x | x | x | x | | x | | x | x | x | 8 |
| Tebuconazole | x | x | x | x | x | | x | x | x | | | 8 |

Table 3: Number of sites and samples for each individual substance and considering them together. It was found totally 13086 cumulative samples (all 4 substances were measured simultaneously).

| | **PNEC (µg/L)** | **Number of sites in Sc3** | **Number of samples in Sc3** |
|---|---|---|---|
| Epoxiconazole | 0.18 | 2087 | 21725 |
| Prochloraz | 0.161 | 2387 | 30250 |
| Propiconazole | 0.095 | 3205 | 42391 |
| Tebuconazole | 1 | 2778 | 32394 |
| 4 substances were measured simultaneously | N/A | 1244 | 13086 (cumulative samples) |

Then, the rate of exceedances was evaluated for the identified 13086 cumulative samples. The results are summarised in Table 4. It was found that the individual rates of exceedances are considerably lower comparing to this estimated by the cumulative risk assessment approach applied for the mixture of 4 azole compounds (Epoxiconazole, Prochloraz, Propiconazole and Tebuconazole) which showed together a rate of exceedances about 8%.

Table 4: Number of exceeding samples and overall rate of exceedances for each individual substance and considering 4 azole substances together according to 13086 cumulative samples (all 4 substances were measured simultaneously) from 3MS.

| | **Total number of samples in Sc3** | **Number of exceeding samples** | **Rate of exceedances (as % from the total number of samples)** |
|---|---|---|---|
| Epoxiconazole | 13086 | 12 (RQ>1) | 0.09 |
| Prochloraz | 13086 | 3 (RQ>1) | 0.02 |
| Propiconazole | 13086 | 39 (RQ>1) | 0.30 |
| Tebuconazole | 13086 | 3 (RQ>1) | 0.02 |
| all 4 substances together | 13086 | 1053 (∑RQ>1) | 8.0 |

| | | | |
|---|---|---|---|
| (cumulative approach) | | | |

**Analytical methods**

Azole compounds can be measured together, thus detected in the same run by using the same analytical method (LC-MS/MS)[8]. The sensitivity of the method is optimal (LOQ < PNEC). Table 5 lists all the available analytical standards required for the for analysis of the proposed azole compounds. To optimise the LC-MS/MS method the single compounds have to be injected to characterise the MS/MS fragments. After optimisation, all standards can be mixed and separated with the HPLC column. They can then be analysed in one LC-MS/MS run. The LOD/LOQ can be calculated from the mixture.

Table 5. List of azole compounds and commercially available analytical standards.

| Azole compound | Type | Price (EUR) | Link |
|---|---|---|---|
| Clotrimazole | VETRANAL™, analytical standard | 70.70 | https://www.sigmaaldrich.com/catalog/product/sial/33894?lang=it&region=IT |
| Fluconazole | Pharmaceutical Secondary Standard; Certified Reference Material | 70.10 | https://www.sigmaaldrich.com/catalog/product/sial/phr1160?lang=it&region=IT |
| Miconazole | Pharmaceutical Secondary Standard; Certified Reference Material | 88.10 | https://www.sigmaaldrich.com/catalog/product/sial/phr1618?lang=it&region=IT |
| Epoxiconazole | PESTANAL®, analytical standard | 58.50 | https://www.sigmaaldrich.com/catalog/product/sial/36848?lang=it&region=IT |
| Imazalil | PESTANAL®, analytical standard | 59.30 | https://www.sigmaaldrich.com/catalog/product/sial/32007?lang=it&region=IT |
| Ipconazole | Dr. Ehrenstorfer (LGC) | 281 | https://www.lgcstanda |

| Azole compound | Type | Price (EUR) | Link |
|---|---|---|---|
| | | | rds.com/IT/en/p/DRE-C14365000 |
| Metconazole | PESTANAL®, analytical standard, mixture of isomers | 89.70 | https://www.sigmaaldrich.com/catalog/product/sial/37909?lang=it&region=IT |
| Penconazole | PESTANAL®, analytical standard | 39.40 | https://www.sigmaaldrich.com/catalog/product/sial/36189?lang=it&region=IT |
| Prochloraz | PESTANAL®, analytical standard | 56.10 | https://www.sigmaaldrich.com/catalog/product/sial/45631?lang=it&region=IT |
| Tetraconazole | PESTANAL®, analytical standard | 176 | https://www.sigmaaldrich.com/catalog/product/sial/37087?lang=it&region=IT |
| Tebuconazole | PESTANAL®, analytical standard | 64.70 | https://www.sigmaaldrich.com/catalog/product/sial/32013?lang=it&region=IT |
| Propiconazole | PESTANAL®, analytical standard | 77.20 | https://www.sigmaaldrich.com/catalog/product/sial/45642?lang=it&region=IT |

**Opinion of the WG Chemicals' experts (MS, EFTA countries and stakeholders)**

After the WG Chemicals meeting held in Brussels on the 15-16 of January 2020, DG ENV asked MS to provide any additional monitoring data, if available, and express their opinion on the substances proposed by the JRC for the WL.

Sixteen MS were in favour of including azole pharmaceuticals in the WL, while two MS were against the inclusion of these substances. One MS expressed doubts regarding the inclusion of these substances and two MS expressed no opinion.

The majority of MS (nineteen MS) were in favour of including the group of azole compounds used as PPP in the WL, while two expressed no opinion and one was against the inclusion. Among the MS in favour, four MS proposed reducing the azole compounds to be monitored

(only metconazole; all except epoxiconazole; only metconazole and prochloraz; epoxiconazole, metconazole and prochloraz).

Finally, two MS (DK and ES) proposed two additional azole compounds used as PPP and biocide i.e. propiconazole and tebuconazole to be included in the WL.

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