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REVIEW ARTICLE

Human and ecological risk assessment of a crop protection chemical: a case study with the azole fungicide epoxiconazole

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Abstract

Conventional risk assessments for crop protection chemicals compare the potential for causing toxicity (hazard identification) to anticipated exposure. New regulatory approaches have been proposed that would exclude exposure assessment and just focus on hazard identification based on endocrine disruption. This review comprises a critical analysis of hazard, focusing on the relative sensitivity of endocrine and non-endocrine endpoints, using a class of crop protection chemicals, the azole fungicides. These were selected because they are widely used on important crops (e.g. grains) and thereby can contact target and non-target plants and enter the food chain of humans and wildlife. Inhibition of lanosterol 14α-demethylase (CYP51) mediates the antifungal effect. Inhibition of other CYPs, such as aromatase (CYP19), can lead to numerous toxicological effects, which are also evident from high dose human exposures to therapeutic azoles. Because of its widespread use and substantial database, epoxiconazole was selected as a representative azole fungicide. Our critical analysis concluded that anticipated human exposure to epoxiconazole would yield a margin of safety of at least three orders of magnitude for reproductive effects observed in laboratory rodent studies that are postulated to be endocrine-driven (i.e. fetal resorptions). The most sensitive ecological species is the aquatic plant Lemna (duckweed), for which the margin of safety is less protective than for human health. For humans and wildlife, endocrine disruption is not the most sensitive endpoint. It is concluded that conventional risk assessment, considering anticipated exposure levels, will be protective of both human and ecological health. Although the toxic mechanisms of other azole compounds may be similar, large differences in potency will require a case-by-case risk assessment

Keywords

Azoles, azole fungicides, endocrine disruption, epoxiconazole, risk assessment

History

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Table of Contents

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Introduction

Risk assessment is widely used as a tool to integrate hazard assessment and exposure quantification to characterize the potential for chemicals in the environment to act as health hazards to humans and biota (Figure 1). Typically, hazard assessment takes a broad approach and is based on a diverse array of biological endpoints that may affect aspects of fitness such as survival, growth and reproduction. New regulatory approaches could place increased emphasis on hazard identification criteria, such as endocrine disruption, as the sole endpoints for regulatory decision making. This approach is in direct contrast to the current risk assessment paradigm which also considers exposure. With pending legislative changes in Europe there will be a replacement of EU Directive 91/414/EEC with Regulation (EC) number 1107/2009 which provides rules governing plant protection products, and would exchange conventional risk assessment for a hazard-based approach to regulation. At present there remains considerable uncertainty as to how to consider endocrine disruption in a regulatory context, and should it be considered as a mode of action or a hazard. For example, there are questions as to whether endocrine disruption merits unique consideration when evaluating specific chemicals and whether evidence of endocrine disruption in the absence of exposure assessment represents an effective endpoint for regulatory decision making. These questions have not so far been addressed. The primary aim of this review was to address this issue, using the azole fungicides as a case study

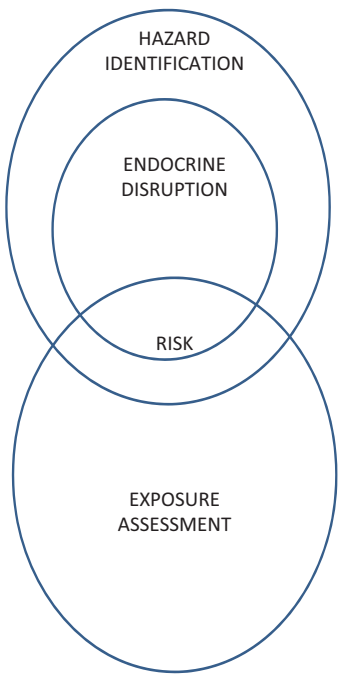

Figure 1. Risk Assessment is a tool to integrate hazard identification with exposure assessment to characterize the potential for chemicals to act as health hazards. Hazard identification criteria, such as endocrine disruption, are receiving increased interest.

The World Health Organization (WHO) in collaboration with the International Programme on Chemical Safety (IPCS) defined an endocrine disruptor as an agent or mixture that ''alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations'' (WHO-IPCS, 2002). A key aspect of this definition is the term adverse which relates to significant deficits in processes that are controlled by the endocrine system. The WHO document specifically stated that endocrine disruption is not a toxicological endpoint but rather a functional change that may lead to adverse effects. Exposure is implicit in this definition and approaches were described in the WHO document for determining causality between exposures to endocrine disrupting chemicals (EDCs) and selected outcomes. More recently, the EU framework for EDCs proposed an *a priori* regulation of chemicals and mixtures that may show some endocrine mediated effects in a variety of experimental systems (*in vitro*, *in silico* and *in vivo*) and further assumed these effects may occur under the default assumption of no thresholds. This is a radical approach and one for which there is considerable debate (e.g. Bergman et al., 2013). This leaves the question of whether identification of a potential endocrine disruptor *per se* provides meaningful information on which to base evidence-based safety assessment and provides better protection than that which already exists in conventional risk assessments. The literature that forms the basis for this review was accumulated to find information on classical toxicity endpoints as well as endpoints that are likely to have resulted because of endocrine disruption, to determine the exposure levels at which these endpoints are caused, and to compare the non-endocrine to the endocrine relevant endpoints to assess whether endocrine disruptive mechanisms would provide a more protective risk assessment.

An azole is a nitrogen-containing five-membered conjugated ring with at least one additional non-carbon atom. The azole fungicides/antifungals are either imidazoles with two nitrogens or triazoles with three nitrogens. Epoxiconazole, the specific pesticide focused upon in this review, is a triazole.

The azoles are a diverse class of compounds that were developed primarily as antifungal agents for use in agriculture and as human/veterinary therapeutics. (This review will use the term fungicide for azoles used agriculturally and antifungal for azoles used as human or animal drugs.) Azoles act by inhibiting the fungal enzyme lanosterol 14α-demethylase, an enzyme in the cytochrome P450 (CYP)

superfamily, specifically CYP51, which produces ergosterol, an important component of the fungal plasma membrane. However, the actions of azoles are not specific to this fungal CYP and as such they may affect other CYPs in non-target species. Several of the CYPs play key roles in hormone synthesis and metabolism in humans and animals, making endocrine disruption a predictable side effect of azole action. Indeed some azoles, such as letrozole, have been developed specifically for therapeutic use as aromatase (CYP19) inhibitors, and which are widely used as an effective treatment for breast cancer prevention/recurrence (Miller et al., 2002). A range of other azole compounds are used therapeutically in humans and animals for a wide range of fungal infections. Other azole compounds are used to control fungal infestations in agriculture, a prime example being epoxiconazole (2*RS*,3*SR*)-1-[3-(2-chlorophenyl)-2,3-epoxy-2-(4-fluorophenyl) propyl]-1*H*-1,2,4-triazole), which is widely used as a crop protection product where it is effective in preventing leaf blotch (*Septoria tritici*) (Paveley et al., 2001) and rust (*Puccinia triticina)* in wheat (Blake et al., 2011). The exposure of biota following agricultural application of epoxiconazole, and the possibility of human exposure from consumption of azole fungicide-contaminated food, raise the possibility of endocrine disruption (e.g. CYP19 inhibition) as an unintended consequence of agricultural practices.

The current risk assessment approach for the agricultural fungicides would protect from the likelihood of off-target endocrine disruption by taking into account the level of exposure to the fungicide in comparison to the levels required to elicit toxic effects. However, the adoption of a hazard-based approach with a focus on endocrine disruption would mean that exposure is not taken into consideration. The consequences of such a regulatory change are uncertain.

Because of both the availability of conventional risk assessments and the possibility of endocrine disruption through CYP inhibition (especially CYP19), this paper will focus on azole fungicides with emphasis on epoxiconazole as a useful and representative example. This review is intended to contribute to the evolving scientific discussion relative to endocrine disruption as an endpoint to address human health and environmental risks from crop protection products. We will summarize the available data that underpin the current risk assessment for epoxiconazole by focusing on the data for hazard assessment and exposure characterization. We will also summarize data on the potential of azole fungicides/antifungals to perturb endocrine function in humans and wildlife, while remaining mindful that the various azoles have different biological potency because of toxicokinetic and toxicodynamic differences. Finally we will critically compare endocrine disruption to other classic adverse outcomes for use as endpoints in risk assessment. While epoxiconazole is used as the primary example, the issues discussed for this compound, and for azole compounds in general, also have relevance for a range of other chemicals released to the environment that have endocrine disrupting properties. A comprehensive search was conducted using the terms epoxiconazole, azole fungicides, toxicology, risk assessment, and mode of action, among other terms, in order to retrieve data that were publicly available either through journals or accessible regulatory documents. Information sources were not added to this review after May 27, 2013.

Molecular targets of azoles

Azole fungicides affect the cell membrane assembly of fungi and yeast by blocking the synthesis of the essential membrane component ergosterol. They do so by competitively inhibiting the enzyme lanosterol 14α-demethylase which leads to ergosterol depletion and the accumulation of lanosterol and other 14-methylated sterols. As a result, the structure of the plasma membrane is disrupted and becomes more susceptible to further damage (Georgopapadakou, 1998; Ji et al., 2000; Sheehan et al., 2000). These changes also alter the activity of several membrane bound enzymes including those involved in nutrient transport and chitin synthesis (Georgopapadakou & Walsh, 1996).

Sterol 14α-demethylase also plays an important role in cholesterol synthesis in mammals (Koltin & Hitchcock, 1997). When azoles are used therapeutically or agriculturally, their antifungal efficacy is attributed to their greater affinity for fungal enzyme than for the mammalian or plant enzymes (Georgopapadakou, 1998). For example, the IC_{50} of epoxiconazole with yeast (*Candida albicans*) CYP51 is 0.22 μM, whereas it is 1.95 μM with human CYP51 (Trosken et al., 2006). Triazole inhibition of CYP51 occurs through binding to the iron of the heme group of the cytochrome, and the binding strength varies with the hydrophilic H-bonding region (Chai et al., 2009; Guan et al., 2010). Through this interaction, the azole molecule occupies the active site of the enzyme and acts as a non-competitive inhibitor (Guan et al., 2010).

Some azole compounds are not specific in binding to the heme moiety of only CYP51 but can inhibit many different CYPs (Kjaerstad et al., 2010; Zarn et al., 2003; Zhang & Mario, 2002). Many studies have focused on CYP19 (aromatase) given its importance in estrogen biosynthesis. Using cell cultures (human and rat granulosa cells, porcine luteal cells, rat pituitary and adrenocortical cells), epoxiconazole has been shown to inhibit production of estradiol, although different sensitivities of effect were observed: human granulosa cells < rat granulosa cells < porcine luteal cells (Wuttke et al., 1995). In further comparison of cultured human and rat granulosa cells, effects of epoxiconazole on CYP19 activity were found at concentrations between 10^{-4} and 10^{-6} M, whereas the therapeutic aromatase inhibitor, letrozole, was active at 10^{-7} M (Wuttke et al., 2001); again, inhibition of CYP19 was more pronounced in rat than in human granulosa cells. The effects on CYP19 are not restricted to mammals as azoles have been shown to inhibit aromatase expression and/or activity in birds, reptiles, amphibians and fish (e.g. Chardard & Dournon, 1999; Richard-Mercier et al., 1995; Vaillant et al., 2001a; Villeneuve et al., 2006)

Using similar approaches and various cell systems, a considerable number of studies have evaluated a range of azoles, including epoxiconazole, for their CYP19 inhibitory activity (Kjærstad et al., 2007; Kjaerstad et al., 2010; Quignot et al., 2012; Villeneuve et al., 2007a; Vinggaard et al., 2000; Zarn et al., 2003). Depending on the cell system and culture

conditions used (e.g. CYP19 substrate used and its concentration), various potencies of effect have been reported, although all such studies demonstrate differences in potency among different azole compounds. This is best illustrated in a representative way by reference to a study that used recombinant human CYP19 in a cell-free system and compared the potency of 22 different azoles under constant conditions (Trösken et al., 2004). This study showed that the potency of different azoles varied across four orders of magnitude (Figure 2). The most potent CYP19 inhibitors were the azoles that are used therapeutically to inhibit aromatase in women with breast cancer (e.g. fadrozole, letrozole), whereas the antifungals and fungicides showed a broad spectrum of potencies. Among the fungicides, prochloraz stood out as the most potent CYP19 inhibitor with hexaconazole the weakest and epoxiconazole having intermediate potency IC_{50} concentrations towards human CYP19 (aromatase) of 0.047 μM, 35 μM, and 1.44 μM, respectively (Trösken et al., 2004). The selectivity of newer generation triazoles such as fluconazole and voriconazole is greatly improved compared to older triazoles (Kale & Johnson, 2005). The biological activity of triazoles depends not only on the docking efficiency to P450, but is influenced by a variety of toxicokinetic factors such as metabolism.

Thus, triazole fungicides may interfere with a broad diversity of CYPs present in plants and animals and this may cause effects in non-target species. As several CYPs play key roles in hormone synthesis and metabolism, endocrine disruption (ED) is a predictable side effect. In toxicity studies of various triazoles, adverse reproductive and/or developmental defects have been observed that may involve ED, indicating the possibility that inadvertent exposure to these compounds may cause endocrine-disrupting effects in humans and wildlife.

Human risk assessment

Toxicity assessment in laboratory animals

Standard toxicity tests of epoxiconazole

The following section describes the results of the toxicity studies used to contribute to the hazard assessment portion of the conventional human health risk assessment for epoxiconazole. Greater detail is provided on observations related to reproductive and endocrine endpoints, along with some mechanistic interpretations related to endocrine disruption. Much of the following information is based on the Draft Assessment Report (DAR), public version (DAR, 2006a).

Based on oral, dermal and inhalation studies in rats, epoxiconazole is of low acute toxicity, with the overall acute oral LD50 set at 5000 mg/kg, the dermal LD50 value above 2000 mg/kg, and inhalation LD50 >5.3 mg/L within 4 h. No local reaction and no skin sensitization were seen in the Guinea Pig Maximization Test (OECD 406) and no irritation to skin and eyes of rabbits in OECD tests.

In dietary exposure studies in rats and mice for 4 or 13 weeks, the liver was the most sensitive target organ, with the NOEL from the 13 week rat studies of 90 ppm (7–8 mg/kg/d). In a supplementary 90 day study in rats, the NOEL was 500 ppm (35 and 41 mg/kg/d in males and females, respectively). In a study with male mice, the NOAEL was <30 ppm (<6 and <9 mg/kg/d for males and females, respectively), and in a further 3 month study in mice, a NOEL of 15 ppm (4 and 5 mg/kg/day for males and females, respectively) was found.

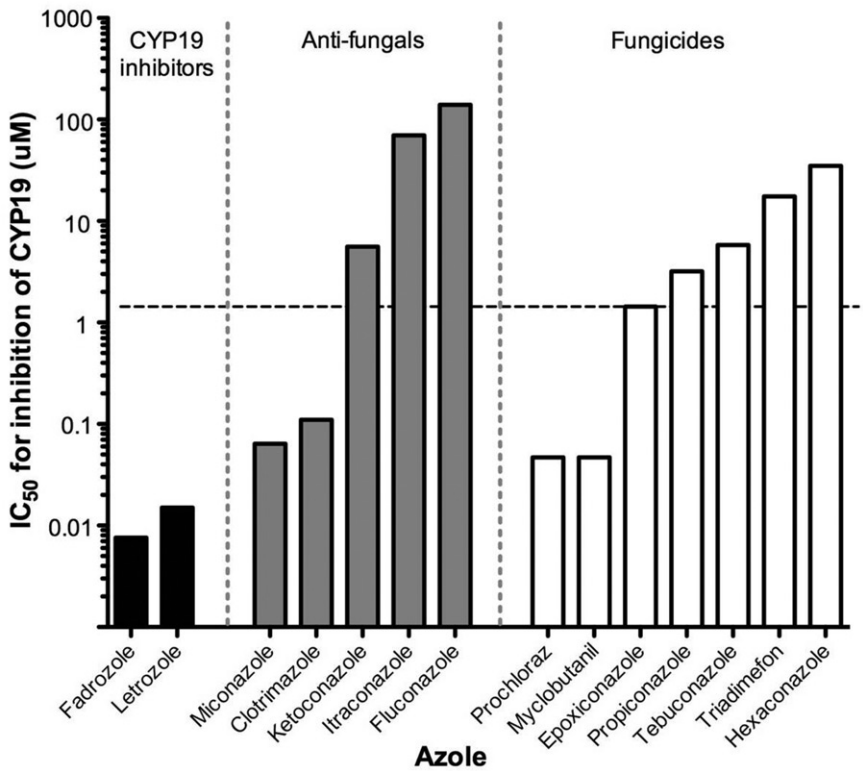

Figure 2. Comparative differences in potency of azoles from different ''classes'' to inhibit human CYP19 activity *in vitro*. This example is derived from the single study by Trösken et al. (2004), which used recombinant human CYP19 in a cell-free system; note also that this study used a wider range of azoles (N = 22) than those illustrated. The differences in potency shown are generally representative of numerous studies in the literature that have used Cyp19 from various species and tissues, although some species differences in susceptibility of Cyp19 to inhibition by azoles are evident, as is discussed in the text.

With the liver as an endpoint, in a dietary 3-month study in dogs, NOELs of 200 ppm (7.3 mg/kg/d) for females and 50 ppm (1.9 mg/kg/d) for males were identified, and in a 12 month dietary study in dogs, a NOEL of 50 ppm (1.6 mg/kg/d) in females and <50 ppm in males was indicated. In another 3-month study, a NOEL of 7.5 ppm (2 mg/kg/d) was identified, based on hematological changes and effects on lipid metabolism. In a supplementary 12-month study the NOEL was 40 ppm (1.1 mg/kg/d).

Extensive in vitro and in vivo tests indicate that epoxiconazole has no mutagenic or genotoxic potential. Similar to the shorter term studies, in long term dietary studies in rats and mice, the liver was the primary target organ for adverse effects plus effects on red blood cell parameters in rats. Inhibition of enzyme(s) involved in the synthesis of steroid hormones was considered the possible mechanism that affected the endocrine system. Increased tumor incidence in female rats (1500 ppm: adrenal gland cortex and ovarian theca granulosa cells) and in mice (males: 500 ppm; females: 1000 ppm; liver tumors) were observed only at dose levels that also resulted in significantly reduced body weights.

In addition to the information presented above that is reported in the DAR (2006a), the US Environmental Protection Agency has also summarized some regulatory data sets in its assessment of epoxiconazole for consideration of tolerances on imported bananas and coffee. This report indicates agreement on the sensitivity of the rodent liver to epoxiconazole administered for 24 months to male rats; liver carcinomas were observed at 1500 ppm but not at 750 ppm (equivalent to 80 and 40 mg/kg/d, respectively) (US EPA, 2005).

Reproductive toxicity of epoxiconazole

Pregnant rats were treated with up to 180 mg/kg epoxiconazole by gavage from days 6–15 post coitum and examined on day 20 (DAR, 2006a). At the highest dosage, signs of maternal toxicity (i.e. impaired food consumption and reduced body weight gain) were found. Placental weights were dose dependently increased and there was a high incidence of fetuses with cleft palates, affecting 90% of the litters in the 180 mg/kg group. In a follow-up study pregnant rats were treated orally with 0 or 180 mg/kg epoxiconazole from days 6–19 post coitum (DAR, 2006a). Decreases in red blood cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration and platelet number, and increased clotting time were observed in treated dams. Blood levels of estradiol, progesterone and prolactin were markedly reduced, while LH was increased. Additionally, there was a high incidence of post-implantation loss and total fetal resorptions. In contrast to the original study, in this follow up study, cleft palate was observed in only one litter in each experimental group.

In a further study, pregnant rats were treated orally with up to 45 mg/kg epoxiconazole from days 6–15 post coitum and dams were sacrificed on day 20 (DAR, 2006a). Epoxiconazole treatment transiently reduced maternal food consumption and impaired body weight gain at 45 mg/kg. At 15 and 45 mg/kg, placental weights were increased, and at 45 mg/kg there was a slight increase in number of fetal resorptions, a marginal increase in post-implantation loss and a marked increase in number of fetuses with skeletal variations, mainly supernumerary ribs.

In a 2-generation dietary study, male and female rats received up to 250 ppm (DAR, 2006a). At the highest dosage there was decreased food consumption and increased liver weight in F0 females and in the F1 parental animals and their progeny, as well as some impairment of reproductive parameters (mating, impregnation and parturition), but all parental animals were fertile. There were some decreases in viability index observed in both generations.

In a dermal study, rats were treated with up to 1000 mg/kg from days 6–15 post coitum (DAR, 2006a). Although the highest dose had no effect on maternal food consumption and weight, there was a significant increase in the number of fetuses with rudimentary cervical and/or accessory 14th ribs.

In an additional prenatal toxicity study, female rabbits were treated orally with up to 80 mg/kg from day 7–19 post-insemination (DAR, 2006a). On day 29 animals were sacrificed and examined. Food consumption was reduced at all dose levels. At the highest dose post-implantation losses and increased numbers of early and total resorptions were found.

To investigate maternal toxicity in more detail, pregnant rats were treated orally from days 6–19 post coitum at dosages up to 75 mg/kg (DAR, 2006a). Decreased food consumption, body weight and platelet numbers were observed, as was an anemic effect which may have resulted in embryo-fetal toxicity from reduced oxygen. Clinical chemistry changes indicated impaired liver function, whereas increased liver weight was seen at the highest dosage only. Fetuses were not examined.

Endocrine effects from epoxiconazole treatment

In a non-guideline study, pregnant rats were treated orally with 0, 15 or 50 mg/kg epoxiconazole from gestation day (GD) 7 until postnatal day (PND) 16 (Taxvig et al., 2007). Pregnancy rate was unaffected but evidence of dystocia (difficult delivery) was apparent at 50 mg/kg. Epoxiconazole had a marked fetotoxic effect and 5 of 7 dams treated with 50 mg/kg were unable to deliver their pups normally. In female offspring, anogenital distance (AGD) was increased, indicating a virilizing effect, although the AGD did not approach male values. However, a subsequent study at similar doses of epoxiconazole did not confirm the AGD increase in female offspring (Taxvig et al., 2008).A potential explanation for the small increase in AGD in female offspring in the earlier study may be that it resulted from the observed, 2-fold increase in maternal testosterone levels which, in turn, may have resulted from a reported 7-fold increase in progesterone levels in the dams. No statistically significant effects on hormone levels were found in GD 21 fetuses, although there was a trend toward lower estradiol in female pups and lower testosterone in male pups.

In a follow-up study, pregnant rats were administered 0 or 50 mg/kg epoxiconazole by gavage from GD 7-21 (Taxvig et al., 2008). Treatment induced post-implantation loss, an increase in late and very late resorptions and

an increase in fetal weight in both sexes. Treated dams had decreased plasma estradiol and increased testosterone. Similar to Taxvig et al. (2007), there was no significant change in measured fetal hormone levels (progesterone and testosterone in fetal plasma and estradiol in ovaries) although a tendency towards a decrease in testosterone was seen.

To determine effects on hormone levels, rats were treated via diet (1500 and 3000 ppm) for approximately 4 weeks or by gavage (200 mg/kg) for 4–6 days (Mellert, 1992). A trend toward increased testosterone and decreased estradiol and a statistically significant increase in androstenedione was found after 4 days of treatment in males. After 4 weeks, androstenedione and follicle stimulating hormone (FSH) showed a significant dose dependent increase and a 40% decrease in corticosterone occurred at the highest dose (3000 ppm). In females after 4 days treatment, significantly increased levels of dehydroepiandrosterone, androstenedione, luteinizing hormone (LH) and FSH were seen together with a decrease in estradiol and corticosterone (the latter not statistically significant). After 4 weeks, changes in the same hormones were seen, the increase in estradiol levels being statistically significant at 3000 ppm; in the latter group an increase in adrenocorticotropic hormone (ACTH) levels was also found. Marked reductions in aldosterone and prolactin were observed in females during proestrus.

The hormonal changes found after treatment of pregnant rats are most readily interpreted as stemming from inhibition of aromatase (Cyp19) activity. Because Cyp19 converts both testosterone to 17β-estradiol and androstenedione to estrone, inhibition of Cyp19 would lead to increased androgens and decreased estrogens. The decreased estradiol level would trigger a feedback response in the hypothalamic–pituitary axis resulting in increased LH and FSH levels. The changes concerning adrenal steroids (corticosterone and aldosterone) and ACTH can be explained by decreased adrenal 11- or 21-hydroxylase activity. Reduced activity of these enzymes would result in decreased production of corticosterone and aldosterone, without affecting testosterone synthesis. The decreased adrenal steroid levels would trigger a feedback response in the hypothalamic-pituitary axis resulting in increased ACTH levels.

Reproductive toxicity of other azole compounds

There have been numerous studies of the effects of exposure to other azole fungicides during pregnancy in rats, using a range of doses. In general, these studies have found similar effects to those found using epoxiconazole, although the dose-dependence and magnitude of effect varies among compounds. This similarity is helpful in identifying common mechanisms of action of azole compounds, for example, inhibition of CYP19. However, we are well aware that the pharmacokinetic/toxicokinetic profile will differ among the various azoles, and the response to any azole will be dependent upon absorption, the efficiency of first pass metabolism as well as metabolism in general, the presence or absence of induction of xenobiotic metabolizing enzymes, and pharmacodynamic/toxicodynamic differences in inhibitory potency toward CYP 19 and other CYP's. Despite these caveats and the fact that there are some differences in adverse outcomes among the various azoles, there is still substantial similarity in the responses observed within the chemical class, strongly suggesting that much of the pharmacological/toxicological response is the result of a common mechanism of action.

In rats, exposure during pregnancy to the potent CYP19 inhibitor letrozole (approximately 0.01–0.04 mg/kg/day from GD 6–16, added to the drinking water) resulted in a dose-dependent increase in post-implantation loss, with 47% loss at the highest dose compared to 7% in controls with no effect on maternal body weight (Tiboni et al., 2008). Viable fetuses also showed a dose-dependent increase in skeletal (vertebral) abnormalities, the magnitude of change being similar to that for post-implantation loss; however, there was no occurrence of cleft palate. The types of skeletal abnormalities were suggestive of delayed ossification (Carney & Kimmell, 2007). In a follow-up study using just the higher dose of letrozole, it was shown that co-treatment of the pregnant rats with estradiol cyclopentyl-propionate (ECP) at 1 or 2 mg/kg/day (by injection) prevented the letrozole-induced post-implantation fetal loss, but did not prevent induction of the skeletal abnormalities (Tiboni et al., 2009). Additionally, letrozole exposure induced a significant increase in placental weight, a change prevented by co-treatment with ECP. Thus, the skeletal abnormalities can be divorced from Cyp19 inhibition by letrozole in these studies as they cannot be prevented by estrogen treatment, whereas the placental weight effects and post-implantation fetal loss can be attributed to estrogen deprivation stemming from Cyp19 inhibition. As discussed below, this pattern of effect is reproduced with other azole compounds when administered to pregnant rats at sufficiently high doses.

Oral exposure of rats from early in pregnancy (GD 0–7) to high doses (>40 mg/kg/d) of hexaconazole (Kumar et al., 2011), myclobutanil, propiconazole (Goetz et al., 2007; Rockett et al., 2006), or tebuconazole (Taxvig et al., 2007), result in one or more of the following adverse pregnancy changes: dystocia and/or increased litter loss, fetal death at birth and/or reduced litter size. For tebuconazole, dose-dependent increases in post-implantation loss and decreased fetal weight on GD21 have been shown (Taxvig et al., 2007).

In rats a significant increase in AGD has been reported in female offspring born to mothers treated during gestation with high doses of myclobutanil (4000 ppm from gestation day 5) (Rockett et al., 2006), or tebuconazole (50 or 100 mg/kg/d) administered from GD 7 (Taxvig et al., 2007). At face value, such effects are consistent with supranormal fetal exposure to androgens that might be expected to result from treatment-induced inhibition of Cyp19. However, the increase in AGD was small (all values well below the normal male range) and there was no reported masculinization of the female external genitalia. Furthermore, at least for doses of tebuconazole and epoxiconazole that caused an increase in AGD, these also generally caused an increase in gestation length, increased post-implantation loss and changes in offspring body weight (decreased at gestation day 21,

increased at birth) (Taxvig et al., 2007), which could be viewed as confounding factors.

The most studied antifungal azole in pregnant rats has been ketoconazole. Exposure during various days of pregnancy to dosages ranging from 12 to 50 mg/kg/day has been shown to induce a range of adverse effects, in particular delayed birth, post-implantation loss or whole litter loss (Cummings et al., 1997; Wolf et al., 1999). These effects are generally attributed to suppression of ovarian Cyp19 (Cummings et al., 1997; Latrille et al., 1989; Watanabe & Menzies, 1985; Wouters et al., 1988), although decreased progesterone production may also occur if treatment is administered early (days 1–8) in pregnancy (Cummings et al., 1997). However, the effects are likely to be age-specific as administration of single doses of 10–100 mg/kg ketoconazole to pregnant rats on day 13 of gestation had no significant effect on post-implantation loss or fetal weight (Dodo et al., 2010). However, treatment of pregnant rats with 25 mg/kg/day ketoconazole on gestation days 12–14 resulted in an almost immediate (GD 15) increase in placental weight that progressed to hypertrophy by the end of pregnancy, although there was no effect on maternal weight (Furukawa et al., 2008). Similarly, miconazole (10 mg/kg) administration to pregnant rats that had been treated with the anti-progestin RU486 on days 6, 9 or 12 of pregnancy exacerbated the abortifacient effects of RU486, an effect associated with suppression of ovarian Cyp19 (Sasaki et al., 1989).

The sequence of events that follows azole treatment of pregnant rats and how this leads, via inhibition of Cyp19 in the ovary, to adverse placental growth/development/function and thus to fetal growth restriction or death (post-implantation loss) or to dystocia is illustrated schematically in Figure 3. This sequence in rodents should be referred to when considering the epidemiological and other data from humans which are detailed later.

Effects of azole compounds in humans and non-human primates

While the perspective of this review is agricultural azoles, there is no literature of which we are aware that describes adverse effects associated with agricultural azole exposure in human populations. There are epidemiological studies that have sought associations between exposure to therapeutic azoles and adverse birth outcomes in humans. Because of the commonality among the therapeutic and agricultural azoles in their inhibition of CYPs, we describe the observations cited in the epidemiological studies as indications of possible adverse reproductive outcomes that azole antifungals/fungicides could induce.

Although most azole compounds share certain features, such as their ability to inhibit certain P450 enzymes, their potential effects in humans will depend on the nature of the exposure (intentional versus unintentional) and the level and timing of exposure. In contrast, animal experimental studies have generally used a range of exposures that extend to high levels that are unlikely to occur in humans, unless exposure is intentional (i.e. therapeutic). Because of this distinction, it is pertinent to review the likelihood of human health effects in the context of likely human exposure, starting with effects of high intentional exposure (therapeutic aromatase inhibitors, therapeutic antifungals) through to low unintentional exposure (agricultural fungicides), as this should provide a more

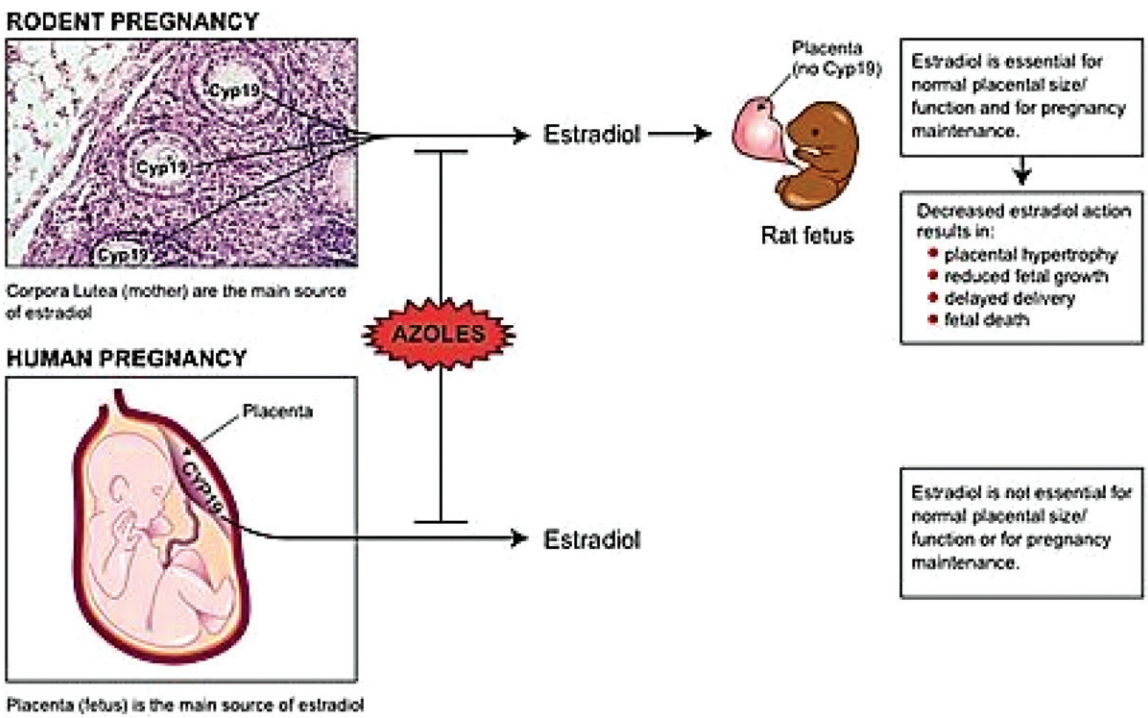

Figure 3. Difference in the site of estradiol production (CYP19 enzyme expression) in rodent pregnancy compared to human pregnancy in relation to the ability of azoles to inhibit CYP19 and cause reproductive toxicity. The presented summary is based on several studies cited in the text, in particular Conley & Hinshelwood (2001).

accurate perspective to the likelihood of adverse effects in situations of unintentional human exposure to azole fungicides.

The capacity of azole compounds to inhibit CYP19 has been exploited via the development of potent aromatase inhibitors (letrozole, anastrozole, fadrozole) by the pharmaceutical industry, for the treatment of estrogen-dependent breast cancer in women. Such compounds are designed specifically to inhibit CYP19, in contrast to azole compounds that have been developed as antifungals or as fungicides, where the inhibition of CYP19 that may occur is an unwanted ''side effect''; this explains the greater CYP19 inhibiting potency of compounds such as letrozole and fadrozole (Figure 2).

Further distinction can be made between azole compounds developed as antifungal therapeutics in humans and azole compounds developed as agricultural fungicides. This distinction rests on the dramatic difference in human exposure that occurs for the two classes of compounds. Thus, use of antifungal therapeutic azole compounds (e.g. itraconazole, fluconazole, miconazole, ketoconazole) results in intentionally high human exposure which can be systemic (e.g. treatment of Coccidioidal meningitis), dermal or vaginal (e.g. *Candida*). In such cases, risk of CYP19 inhibition is lower than for therapeutic CYP19 inhibitors (e.g. letrozole) because of (a) generally lower potency for CYP19 inhibition (Figure 2), and (b) in the case of topical antifungal use, systemic exposure will also be low. Nevertheless, in both instances, human exposure is intentional and, as both vaginal and systemic fungal infections tend to occur more commonly in pregnancy, fetal exposure is likely to occur. In contrast, human exposure to the fungicidal azole compounds used in agriculture (e.g. epoxiconazole, hexaconazole, imazalil, propiconazole, prochloraz, triadimenol) is entirely unintentional. Under normal conditions, it would be expected that exposure to such compounds would be very low for the general population (food residue exposure only) and somewhat higher for applicators (application + food residue exposure).

In the following sections, the consequences of pregnancy exposure to azoles in humans and primates are reviewed. For the treatment of estrogen dependent breast cancer the potent aromatase inhibitors letrozole, anastrozole or fadrozole are used. Although such therapy is contra-indicated in human pregnancy, exposure may occur peri-conceptually due to increasingly common use of letrozole for ovulation induction in women with polycystic ovarian syndrome or with unexplained anovulation (see Casper & Mitwally, 2011 for review). In a study of 514 offspring born to mothers treated with letrozole, the incidence of congenital malformations was 2.4%, which was somewhat lower than that found in the control group in which ovulation induction was via a more standard treatment, clomiphene citrate (Tulandi et al., 2006). There are no reports of cleft palate following pregnancy exposure to therapeutic CYP19 inhibitors in humans/non-human primates. However, this is not really comparable to the rat studies discussed earlier, as even in the worst case scenarios, exposure to letrozole would have been at around the time of implantation and quite transient and not during pregnancy *per se*.

Albrecht et al. (2000) treated baboons with letrozole (2 mg/day, equivalent to ~0.13 mg/kg/day) during various stages of pregnancy and found that treatment initiated during the first, but not during the second, trimester of pregnancy increased the miscarriage rate up to 50%, a change prevented by co-treatment with estradiol. Such an effect is in keeping with observations in the rat Albrecht et al. (2000) discussed in Section 3.1.2. Whether a similar effect would occur in human pregnancy is uncertain as there are no available direct data on equivalent exposure to letrozole, although there are various pieces of indirect data that might suggest a lower susceptibility of human pregnancy to early lowering of estradiol levels (discussed in Albrecht et al., 2000). Probably the most direct data derive from studies in which pregnant women with fungal infections have been treated with high doses of antifungal azoles (with anti-CYP19 activity) during the first trimester of pregnancy, and these show no evidence for increased miscarriage rate (discussed below). It should be noted that there was no report of cleft palate in the baboon study following pregnancy exposure to letrozole (Albrecht et al., 2000).

There are a number of epidemiological studies that report on adverse birth outcomes from therapeutic azole antifungal use during pregnancy. Antifungal medicines provide the next tier in terms of human exposure to CYP19 inhibition by azole compounds; exposure is intentional, is high to moderate but CYP19 inhibition is a side effect rather than being the objective of treatment, in contrast to letrozole. Moreover, because fungal infections are common, and especially so in women (vaginal fungal infections), treatment often encompasses some portion of pregnancy. The primary motivation for these studies was to ascertain if such exposure induced craniofacial and skeletal abnormalities, but in the context of CYP19 inhibition the concern would be whether such exposure would induce miscarriage, preterm birth and/or reduced birth weight, changes reported in experimental rodent studies as described earlier (Figure 3).

A review by Giavini & Menegola (2010) summarizes the more prominent publications related to pregnancy outcomes following azole antifungal exposure in the first trimester. Most studies found few, if any, statistically significant outcomes of congenital abnormalities in the azole-exposed groups compared to matched controls; the data in these studies resulted from pregnant women taking topical or oral doses of the azole antifungals at the recommended dose levels and durations. In a few cases there were statistically significant differences between the pregnancy outcomes of the treated compared to the controls, but the numbers of cases were small, and there were a number of identified confounders that could have influenced the results. Therefore none of these epidemiological studies provided convincing evidence of azole antifungal-induced congenital abnormalities.

One of the sources of information on pregnancy outcomes of women exposed to antifungals and other drugs is the series of publications resulting from epidemiological analyses by Czeizel and colleagues using data from the Hungarian Case-Control Surveillance of Congenital Abnormalities (HCCSCA), a large population-based study (Czeizel et al., 2001). The HCCSCA is reported to be the largest case-control

dataset of its nature in the world (Czeizel, 1997). Information was derived from antenatal log books supplied by the mothers or from other medical records or from survey questions given to the mothers. It included data from 38 151 pregnant women whose offspring did not display congenital abnormalities and, for most of the epidemiological analyses, was compiled from 1980–1996; this number of controls represents 1.8% of all births in Hungary during that period. There were 22 843 pregnant women who had offspring with congenital abnormalities during this period, and they constituted the case group. Frequently, two controls were matched to each case for the analysis.

For investigation of the imidazole econazole, 68 (0.3%) of the cases with congenital abnormalities had been treated versus 122 of the controls (also 0.3%); treatment was via a vaginal suppository at 150 mg/day for 3 days. The crude prevalence odds ratio (POR) was calculated to be 0.9 (95% confidence interval 0.7–1.3), suggesting no evidence for econazole being teratogenic if it was taken during the second and third months of pregnancy (Czeizel et al., 2003). A similar analysis was performed for the imidazole miconazole which was used as a cream. In the control group 46 (0.12%) and in the case group 24 (0.11%) had been treated with miconazole at 200 mg/day, yielding a POR of 0.9 (0.6–1.6). Thus also for miconazole, no evidence of teratogenicity was observed (Czeizel et al., 2004b).

An early study of the imidazole clotrimazole revealed no evidence of teratogenicity (Czeizel et al., 1999). Clotrimazole was administered as either a 100 mg/day vaginal tablet for 6 days or a 200 mg/day vaginal tablet for 3 days or 20 g of a cream containing 200 mg clotrimazole twice daily for 4 days. The study actually suggested a protective effect of clotrimazole in preventing preterm birth-related undescended testes in the male offspring of the treated women. The researchers performed a follow-up analysis to test their hypothesis that clotrimazole can prevent preterm birth (Czeizel et al., 2004a). Of the 38 151 women of the control group (i.e. not having offspring with congenital abnormalities), 3077 (8.1%) used clotrimazole, and those who did had a significant reduction in preterm births. An additional follow-up study investigated the prevalence of preterm births in a group of women who were treated with a combination of metronidazole and miconazole, which comprised 2.2% (846 women) of the control group. The treated group had a prevalence of 9.5% for preterm births, while the untreated group had a prevalence of 9.2%, so this combination did not reduce preterm births as did clotrimazole (Kazy et al., 2005b). Similar analysis with the same large dataset was performed for ketoconazole (Kazy et al., 2005a, b). Ketoconazole was administered orally in 200 mg tablets daily for 5–10 days. Six offspring from cases of congenital abnormalities (0.03%) and 12 offspring from the controls (0.03%) had mothers who were treated with oral ketoconazole; these data yielded a POR of 0.85 (0.3–2.2). Therefore there was no association between ketoconazole exposure and congenital abnormalities.

While the above studies indicated no adverse teratogenic effects of medical use azole antifungals during the second and third months of pregnancy, there were two studies arising from the Hungarian dataset that suggested an association between azole antifungal exposure and congenital abnormalities. The first was on the use of metronidazole and an association with hydrocephalus (Kazy & Czeizel, 2005). The metronidazole was in a vaginal suppository at 500 mg and was recommended for use daily for 10 days, but the recorded mean durations were 7.8 and 8.2 days for cases and controls, respectively. The POR for hydrocephalus was 10.7 (1.1–104.5), the wide confidence intervals resulting from there being only 5 cases. The authors indicated that the results on metronidazole use might have been subject to recall bias because the occurrence of hydrocephalus is so traumatic that mothers might be more inclined to identify a possible exposure in their pregnancies than would mothers of healthy babies. Therefore the authors state that these results are only a signal of a possible association between use of metronidazole and hydrocephalus, and not a definitive conclusion of association. From the perspective of the present review, it is unclear how hydrocephalus would be related to CYP19 inhibition or how hydrocephalus would be a logical outcome of craniofacial abnormalities.

A second study resulting from this same dataset was an analysis of metronidazole and miconazole, with recommended treatment of 100 mg of each applied vaginally daily for 10 days, and actual duration of 8.8 and 8.7 days for cases and controls, respectively. The analysis indicated a POR for syndactyly and polydactyly of 6.0 (2.4–15.2). The authors indicated that these results may also have been subject to recall bias and that these results also are only a signal for the possible association of metronidazole plus miconazole exposure with polydactyly and syndactyly (Kazy et al., 2005b). An earlier study from this research group was published on oral metronidazole using data from 1980–1991 that involved somewhat fewer subjects (30 663 controls and 17 300 cases) (Czeizel & Rockenbauer, 1998). Analysis indicated a slightly greater use of metronidazole in the second and third months of pregnancy in 9 cases with cleft lip (occurring with or without cleft palate), but the conclusion was not confirmed by comparison of cleft lip cases (with or without cleft palate) to the total control group. Again the authors indicated that recall bias might have influenced the results, and the authors concluded that metronidazole during pregnancy was not a teratogen.

In addition to the very large datasets from Hungary described above, a number of other studies have been published from several different countries, most using a considerably smaller dataset. An epidemiological study in the United States evaluated pregnancy outcomes for 234 women exposed to fluconazole, 492 exposed to topical fluconazole, and 88 exposed to an azole that was not fluconazole. The outcomes of these three groups of individuals were compared to outcomes of 1629 individuals not exposed to any of these agents. Relative risks for offspring with a congenital disorder were 1.1 (0.4–3.3), 2.1 (0.7–6.8) and 0.6 (0.2–1.6), respectively. Therefore, there was no evidence that fluconazole exposure during the first trimester of pregnancy resulted in adverse pregnancy outcomes (Jick, 1999).

A prospective cohort study on the triazole itraconazole was conducted using data from a database supplied by the Belgian manufacturer on 156 women orally exposed during pregnancy who had live births and a group of 187 controls from a Canadian database who were not exposed. Although it is

unclear whether these groups were similar, malformation rates were 3.2% in the exposed group and 4.8% in the control group, yielding a relative risk of 0.67 (0.23–1.95). The rate of pregnancy loss was higher in the exposed group: relative risk 1.75 (1.47–2.09). Birth weight in the exposed group was lower but this was not considered clinically significant. The following were all similar between the exposed and the control groups: gestational age at birth, Apgar scores at 1 and 5 minutes, rate of preterm deliveries, and neonatal complications. Therefore itraconazole was considered not to be teratogenic (Bar-Oz et al., 2000).

An additional prospective cohort study on itraconazole was conducted in Italy, with data from 206 women with first trimester exposure to itraconazole and 207 unexposed controls. No significant differences were observed in major congenital abnormalities between exposed and control groups: 3 of 162 (1.8%) and 4 of 190 (2.1%), respectively. There were no differences in the rates of vaginal delivery, premature births, or high or low birth weights. However the rates of live births, spontaneous abortions and induced abortions were higher in the exposed than in the control group. The authors concluded that itraconazole exposure did not lead to an increase in major congenital abnormalities (De Santis et al., 2009). An Italian prospective cohort study of fluconazole provided an analysis of first trimester exposure of 226 women compared to a reference group of 452 unexposed women. There were similarities between the two groups in rates of miscarriage, stillbirths and congenital anomalies, neonatal complications and growth of neonates. The dosage range was 100-2100 mg, with a median of 200 mg. The authors concluded that first trimester exposure did not increase adverse pregnancy outcomes, although there was an increase in induced abortions in the exposed group (Mastroiacovo et al., 1996).

A population-based study on fluconazole conducted in Denmark analyzed data from 121 women exposed to low doses and 13 327 women in the reference group. There was no increase in malformations in the exposed group (4 of 121, 3.3%) compared to the control group (697 of 13 327, 5.2%) yielding an odds ratio of 0.65 (0.24–1.77). In addition, there were no statistically significant differences between exposed and controls in preterm deliveries (OR 1.17; 0.63–2.17) or low birth weights (OR 1.19; 0.37–3.79). Therefore the authors concluded that a single dose of fluconazole did not result in adverse pregnancy outcomes (Sorensen et al., 1999).

Lastly, a publication describing case reports from the United States indicated adverse pregnancy outcomes from high dose exposures to fluconazole during pregnancy (Pursley et al., 1996). These case reports described situations of severe coccidioidomycosis infections from *Coccidioides immitis* resulting in meningitis and requiring extraordinarily high dosing of the azole. The abnormalities that resulted were mostly craniofacial, skeletal and cardiac, and were similar to those seen in animal tests involving high dose exposure to certain azoles, as described earlier. The first case was a woman on therapy of fluconazole at 800 mg/day whose pregnancy resulted in the birth of a male infant who survived with multiple congenital defects. The second case was a woman with coccidioidomycosis that resulted in meningitis for which she was given oral fluconazole at 400 mg/day. Her first and third infants, from pregnancies in which she was compliant to the fluconazole regimen, had multiple severe congenital defects, while her second infant, from a pregnancy when she was not compliant to the fluconazole regimen, did not have malformations. While these cases are only anecdotal, they do suggest that in humans the teratogenic effects appear to be dose-related since this second woman's pregnancy outcomes were related to the compliance-dependent blood levels of fluconazole.

In summary, there have been some epidemiological studies on therapeutic antifungal azoles used during pregnancy, some of which involved a large number of women, and most of the evidence is that the antifungal azoles do not cause developmental defects. The evidence that is suggestive of developmental defects is from extremely high dose exposures or from very small numbers of cases. Therefore the relatively high levels of intentional azole exposure during drug therapy display little to no evidence of inducing congenital abnormalities in human infants, concurring with King et al. (1998) who concluded that there was minimal risk to human pregnancy from such treatments. There were no studies of which we are aware on any agricultural fungicides. Therefore the literature base does not support teratogenicity as a relevant human toxicity of azole exposure.

The studies discussed above also show little or no evidence for adverse effects of human exposure to azole antifungals related to CYP19 suppression, in contrast to rodent studies (i.e. fetal loss, growth restriction, etc.; Figure 3). In this regard, it should also be recognized that comparison of human data involving treatment with azole antifungal compounds in pregnancy with corresponding rodent treatment data is confounded by the fact that in the humans there is a vaginal infection that triggers treatment and such infections are important risk factors for premature delivery. In contrast, in the rat experimental studies there is no predisposing effect due to infection. The fact that some studies have shown that intra-vaginal exposure of women to metranidazole (McDonald et al., 1997; Morales et al., 1994) or clotrimazole (Czeizel et al., 2004a) during pregnancy (usually for <2 weeks), reduced the rate of pre-term births could therefore simply reflect the effectiveness of the antifungal therapy, as vaginal infections in women in pregnancy are the most important established risk factor for pre-term birth.

Mechanisms of developmental adverse outcomes

Two critical observations arising following epoxiconazole treatments of pregnant laboratory rats are fetal resorptions and cleft palates. The literature supports the conclusion that the mechanisms of azole induction of post-implantation losses/fetal resorptions and cleft palates are different, the former being caused by Cyp19 inhibition and the consequent endocrine disruption resulting from inadequate estrogen to support pregnancy. This conclusion is based on studies in which estrogen replacement was able to prevent the reproductive but not the skeletal (e.g. cleft palate) effects induced by azole exposure (Tiboni et al., 2009). Therefore, cleft palates do not seem to result from Cyp19 inhibition, and instead the evidence points to involvement of retinoic acid or TGFβ3; if true, then cleft palates do not arise from an

endocrine disruption mechanism. Below the evidence from the literature for these two mechanisms is presented.

CYP19 inhibition: relationship to post-implantation fetal loss

Estrogens are important for the maintenance of pregnancy in both rodents and humans/primates (Elbrecht & Smith, 1992), but the degree of dependence (more important in rodents than in humans) and the source of estrogens are fundamentally different. In rodents, pregnancy estrogen derives from the corpus luteum (i.e. from the mother) whereas in humans/primates it derives primarily from the placenta (i.e. from the fetus) (Figure 3). Therefore the consequences of CYP19 inhibition in pregnancy may be different in rodents versus humans, and such differences need to be taken into account when extrapolating from rodent effects to humans (Figure 3).

As it is estrogen from the ovary (corpora lutea) in rodents that maintains pregnancy (Figure 3), a further logical conclusion would be that the adverse pregnancy effects of azoles in rodents result from effects in the dams not in the fetuses. Independent demonstration that it must be Cyp19 inhibition by azoles in the dam that causes placental/fetal disruption in rodents comes from studies of *Cyp19* knockout mice (Fisher et al., 1998). In this comparison it is important to remember that it is the offspring that carry the null mutation, not the dam in which Cyp19 function will be normal. Male and female *Cyp19* null mice are born with normal Mendelian frequency, indicating that there is no fetal loss resulting from absence of fetal (or placental) *Cyp19*; the latter finding thus contrasts with the fetal losses, etc., that are found after in vivo exposure of pregnant rats to azoles that inhibit Cyp19 activity, as detailed above. In vivo azole treatment will presumably have inhibited Cyp19 in both dams and fetuses, so the difference in outcome compared with *Cyp19* knockouts is presumably attributable to inhibition of maternal Cyp19 activity. Whether dams carrying *Cyp19−/−* mice are masculinized, as occurs in women carrying babies with non-functional *CYP19* (Zirilli et al., 2008b), has not been reported. As mice/rats do not express Cyp19 in the placenta, in contrast to the human (Conley & Hinshelwood, 2001; Sasaki et al., 1989), it is unlikely that supranormal androgen exposure of the dam will occur in *Cyp19−/−* mice as the main source of estrogen in rodent pregnancy is from the corpora lutea of the dam (Figure 3), within which normal Cyp19 activity will be present (and thus no elevation of androgens will occur). In humans with *CYP19* null mutations there is overt masculinization of the female offspring (Zirilli et al., 2008b), whereas this masculinization does not appear to be the case in *Cyp19−/−* mice, although there may be larger clitoral glands (Fisher et al., 1998).

Inactivating mutations in the *CYP19* gene (in the fetus), which means that there is a complete absence of both fetal and placental aromatase activity, appears to have no effect on pregnancy progression or birth weight of affected offspring. Evidence for this comes from studies reporting on 15 fetuses (8 female, 7 male) with an inactivating mutation in the *CYP19* gene, which showed the babies were delivered of normal weight at term, see Zirilli et al. (2008a). In several of the pregnancies where there were mutations to aromatase gene, monitoring established that placental size and fetal growth were completely normal (e.g. Deladoey et al., 1999; Mullis et al., 1997), which contrasts with what is observed in rat experimental studies involving exposure to azole CYP19 inhibitors (Figure 3). It cannot be discounted that some human fetuses with inactivating mutations in the *CYP19* gene might have miscarried, but this seems unlikely to have occurred at greater than normal frequency. These observations demonstrate that there may be fundamental differences in the importance of CYP19 activity during pregnancy in the rat versus the human, a conclusion supported by data with other azoles, and consistent with understanding about differences in hormonal dependence of pregnancy in the rat (primarily estrogen-dependent) versus human (primarily progesterone-dependent). Female babies born to mothers with inactivating mutations in *CYP19* present with pseudohermaphroditism and exhibit variable degrees of virilization (e.g. cliteromegaly, fusion of labia) as a consequence of the elevation in fetal androgen levels that occurs because of the decreased/absent conversion of androgens to estrogens by CYP19 (primarily in the placenta). Additionally, there is always evidence for virilization of the mother during pregnancy (e.g. hirsutism, acne, cliteromegaly, deepening of voice, masculinizing changes to the face) when carrying babies of either sex with inactivating mutations in *CYP19*, and this is viewed as an alerting feature of such rare pregnancies (Zirilli et al., 2008b). Once the child with an inactivating *CYP19* mutation is born, the masculinizing effects on the mother dissipate, demonstrating the fetal/placental origin of the masculinizing hormones. There are no reports of craniofacial abnormalities in children born with inactivating mutations of *CYP19* (Zirilli et al., 2008b).

In summary, azoles can clearly inhibit CYP19 in humans as in rodents, based on in vitro studies, but there is no evidence that this has any consequence in humans in vivo. This is because continuation of pregnancy in humans is not dependent on estradiol production by the corpus luteum as in rodents, nor is it dependent on estradiol production by the placenta (the predominant source of pregnancy estrogens in humans) as inactivating mutations of placental *CYP19* in humans have no effect on pregnancy progression or duration, although it does have consequences for the mother (virilization). Therefore, the effects of azoles on pregnancy in the rat do not appear to be human health-relevant, a conclusion in keeping with the absence of any reported adverse effects of therapeutic fungicides on pregnancy continuation/duration in humans. Further, there are no reports of virilization of mothers in pregnancy who have been exposed to antifungal azoles.

Mechanism(s) for induction of cleft palate/skeletal defects by azoles

As detailed in the toxicological studies above, exposure of pregnant rodents to certain azoles can induce skeletal abnormalities, in particular cleft palate in the offspring. One possibility is that the skeletal effects stem indirectly from Cyp19 inhibition, with the resulting placental hypertrophy leading to fetal growth deficits and increased azole transfer across the placenta. This possibility cannot be discounted, but

the evidence that estrogen replacement is able to prevent azole-induced placental hypertrophy and the associated fetal losses, whereas it does not prevent the skeletal abnormalities points towards different mechanisms for the reproductive and skeletal effects (Marotta & Tiboni, 2010; Tiboni et al., 2009). Consequently, two alternative mechanisms have been suggested to account for the skeletal effects of azole compounds, inhibition of glucocorticoid production or over-production of retinoic acid (RA; vitamin A).

Glucocorticoids and cleft palate induction: effects of azole compounds

Dietary exposure to epoxiconazole (3000 ppm) for 4 weeks has been shown to result in a 40% reduction in corticosterone levels in rats (Mellert, 1992). Exposure of the human H295 adrenocortical cell line to prochloraz, ketoconazole or imazalil, or mixtures of these compounds, has also been shown to inhibit cortisol production with an IC_{50} of ~0.1 μM (Ohlsson et al., 2010). In keeping with these observations, glucocorticoid co-administration has been shown to alleviate ketoconazole-induced cleft palate in rats (Amaral & Nunes, 2009), implicating deficient glucocorticoid action as the underlying mechanism, i.e. an endocrine disrupting mechanism. However, there is no supporting evidence for such a pathway, indeed quite the reverse. For example, several studies in humans have shown that exposure to exogenous corticosteroids up to about 12 weeks' gestation results in about a 3-fold increase in risk of oral clefts in the resulting babies (Carmichael et al., 2007; Park-Wyllie et al., 2000; Pradat et al., 2003). Moreover, this has been modeled in mice exposed gestationally to dexamethasone (He et al., 2010). There is no corresponding evidence that lack of glucocorticoids leads to development of cleft palate.

Therefore, an indirect mechanism must underlie the ability of glucocorticoids to prevent induction of cleft palate by ketoconazole in rats (Amaral & Nunes, 2009). Glucocorticoids affect many different cellular processes, but the effects most relevant to oral cleft induction may be their ability to suppress TGFβ subtypes (Barnett et al., 2011; Derfoul et al., 2006). TGFβ family members, in particular TGFβ3, play a fundamentally important role in palate development. TGFβ family members are expressed age- and cell-selectively during palatogenesis and are essential for its completion (Figure 4), as illustrated by studies of TGFβ knockout mice (Taya et al., 1999). Thus, TGFβ1$^{-/-}$ mice do not develop cleft palate whereas 100% of TGFβ3$^{-/-}$ mice do (they also die shortly after birth) and 23% of TGFβ2$^{-/-}$ mice develop cleft palates. Increased risk of cleft palate as a result of folate deficiency may also stem from reduction in TGFβ3 (Maldonado et al., 2011).

In wound healing, glucocorticoids inhibit expression of TGFβ1 and TGFβ2 (and TGFβRII) whereas expression of TGFβ3 and TGFβRI are increased (Frank et al., 1996). Similarly, glucocorticoid induction of fetal lung maturation is associated with marked up-regulation of TGFβ3 expression (Wang et al., 1995). Therefore, one possibility is that glucocorticoid treatment up-regulates TGFβ3 in the developing palate (Figure 4), based on its effects in other tissues. As there is some evidence that certain azoles may

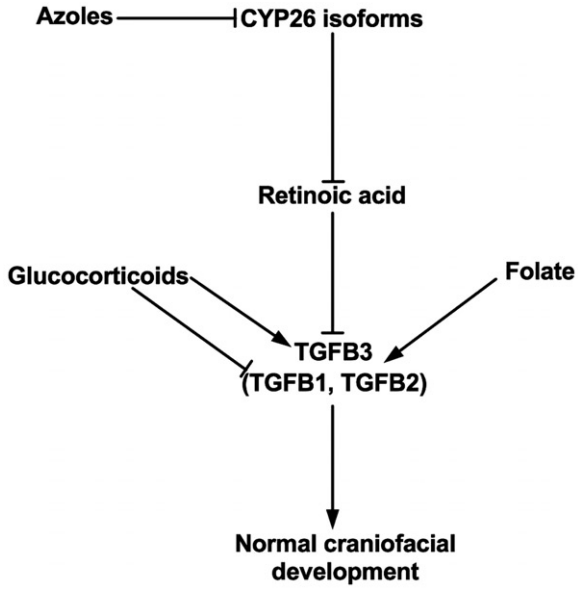

Figure 4. Schematic diagram to illustrate pathways that may explain the ability of azoles and glucocorticoids to differentially affect craniofacial development, including palatogenesis, in animal experimental studies and, by extrapolation, in humans. TGFβ family members are expressed age- and cell- selectively during palatogenesis and are essential for its normal completion. Azoles most likely impact this pathway indirectly via modulation of expression of CYP26 isoforms (eg CYP26B), which leads to altered expression of one or more TGFβs that may lead to impaired craniofacial development. Glucocorticoids can differentially affect expression of TGFβ isoforms in numerous tissues, and this ability may enable it to ''correct'' deficiencies in TGFβ expression as a consequence of azole exposure. The scheme integrates information from numerous studies that are cited in the text, in particular Menegola et al. (2006) and Ackermans et al. (2011).

suppress expression of TGFβ1, TGFβ2 and/or TGFβ1R (Menegola et al., 2006), it is speculated that glucocorticoid induction of TGFβ3 might counteract such effects, assuming that TGFβ3 could substitute for reduced expression of TGFβ1 and TGFβ2. However, this possibility remains to be tested.

Retinoic acid (RA; vitamin A) and cleft palate; effects of azole compounds

RA plays a crucial age- and cell-specific role in cranio-facial morphogenesis, including palatogenesis. Under- or over-exposure to RA at specific fetal ages can disrupt these processes and cause teratogenic effects, including the induction of cleft palate (Lammer et al., 1985; Mahmood et al., 1992; Menegola et al., 2006). Induction of cranio-facial malformations by over-exposure to RA is similar to that caused experimentally by certain triazoles when exposure occurs during early developmental stages (Menegola et al., 2006; Marotta & Tiboni, 2010). Consistent with this, exposure of cultured mouse (Tiboni, 1993) or rat (Menegola et al., 2000) embryos, to various azole compounds (fluconazole, triadimefon, triadimenol) results in impaired branchial arch morphogenesis, an effect that can be additive (Menegola et al., 2004).

Under normal conditions, the local levels of RA in the embryo are regulated in several ways, the most important

being via its catabolism through the activity of CYP26 isoforms (Abu-Abed et al., 2001; Reijntjes et al., 2005). Other mechanisms, for example sequestering to binding proteins such as cellular retinoic acid binding proteins 1 and 2 (CRABP1 and 2), may also contribute to regulation (Figure 4). Inhibition of the normal activity of CYP26 enzymes in a particular tissue, such as the developing head, would therefore result in over-exposure to RA (Abu-Abed et al., 2001; Reijntjes et al., 2005).

The most enlightening study was by Mineshima et al. (2012) who exposed pregnant rats to single doses of either RA or ketoconazole on specific gestational days and then evaluated induction of cleft palate among other skeletal effects. These showed two separate windows of RA-sensitivity, the most important being gestation days 8–10 and the second window at days 12–14. In contrast, ketoconazole only induced cleft palate during exposure from days 12–14 and had no effect during days 8–10. This demonstrates that the mechanisms of cleft palate induction by RA are likely to be different at days 8–10 versus days 12–14, and that only the latter is affected by ketoconazole. Similar age-dependent induction of cleft palate in mice by itraconazole has also been shown (Tiboni et al., 2006). Based on differential importance of TGFβ2 and TGFβ3 in palatogenesis (Taya et al., 1999), it could be postulated that TGFβ3 plays the key role during days 8–10 whereas TGFβ2 is the main player at days 12–14 in rats. This would fit with the evidence that certain azoles can reduce expression of TGFβ1, TGFβ2 and TGFβ1R (Menegola et al., 2006) (see Figure 4). Although suppression of TGFβs may be the change induced by azoles that directly impacts palatogenesis, this effect may stem from altered local RA exposure due to down-regulation of Cyp26B (Menegola et al., 2006). This would lead to increased RA action and thus lead to TGFβ suppression (Figure 4; see also Ackermans et al. (2011)). This could be related to age-dependent differences in expression of Cyp26A and B isoforms (Reijntjes et al., 2005). However, direct study of Cyp26B expression in mice exposed to a teratogenic dose of fluconazole reported increased rather than decreased expression (Tiboni et al., 2009), although the authors suggested that this change may have been secondary to increased RA exposure.

Assuming that the mechanisms proposed above are correct, and that the induction of cleft palate in rodents occurs due to local over-exposure to RA in the fetal head, then the logical conclusion is that cleft palate induction by azoles stems from effects within the fetus, rather than in the mother and via a non-endocrine mechanism. Thus it contrasts with the effects of azoles on Cyp19 (as discussed above) in rodents where the important effects are in the mother not the fetus, and where the mechanism involves endocrine disruption. It should be mentioned that one study has shown that RA can suppress CYP19 in MCF7 cells in vitro (Ciolino et al., 2011). Although this might be considered a potential way of interlinking the mechanism for cleft palate induction by azoles with their inhibition of CYP19, this is unlikely to be the case for two reasons. First, azoles can clearly inhibit CYP19 directly, as detailed earlier, and second, it seems unlikely that azole-induced local elevation of RA within the fetal rat head could lead to sufficient over-exposure of the maternal ovary to RA to inhibit CYP19.

Human exposure to azoles

Human exposure from therapeutic applications

Human exposure to azole compounds results from their therapeutic use (dermal and oral applications) and their use as fungicides in agriculture as well as in veterinary medicine. The levels of exposure from therapeutic azoles are in the hundreds of mg levels over the course of several consecutive days for routine vaginal yeast infections, as was discussed above. These therapeutic levels translate to dosages of low mg/kg/day and did not yield evidence of adverse pregnancy outcomes. The very high doses for extended periods of time to combat meningitis, that did result in congenital malformations, translate to about 10 mg/kg/day.

Human exposure from agricultural occupational applications

A study by Ramwell et al. (2005) investigated the residues of epoxiconazole that would occur on the external surfaces of spray equipment that would be available for contact by the agricultural workers. The researchers used cotton gloves as samplers for typical agricultural tasks of field sprayer operators. Three activities were investigated: entering into and working within the sprayer cab; general handling of the sprayer equipment; and maintenance activities for the sprayer. Epoxiconazole was one of thirteen pesticides analyzed. Thirteen farms of various sizes in England were monitored on two occasions each. Epoxiconazole, along with the other two triazoles monitored (tebuconazole and flusilazole), were the pesticides detected most often on the glove samplers. Residues of epoxiconazole were between 0.1 and 1 mg per glove pair for most samples. The authors expressed their results in terms of the proportion (as percentages) of the samples that would have required over 12 hr of an occupational activity to achieve the Acceptable Operator Exposure Level (AOEL), i.e. 0.02 mg/kg/day for epoxiconazole. Therefore the higher the percentage, the more likely that conduct of this activity would not achieve the regulatory limit (AOEL) in a typical workday. These percentages were 83, 72 and 39, for the cab activities, the general handling and the maintenance activities, respectively. Thus the only activity that yielded substantial exposure in a typical workday exposure was the maintenance activity, which would be predicted since there would be much worker contact with the vehicle and sprayer equipment. The more routine worker activities (i.e. cab activities and general handling) would not yield residues approaching the regulatory limit unless the activities were conducted for a very long period of time exceeding a typical work day.

However it should be noted that a more conservative AOEL value of 0.008 mg/kg/day was derived for epoxiconazole during the EU-Review as published by EFSA (2008). This value derives from the NOAEL of the 12-month dog study (1.6 mg/kg/d) applying a safety factor of 100 and a correction factor of 50% for oral absorption. This newer AOEL, derived after the publication of the Ramwell et al. (2005) paper, is 2.5-fold lower than the AOEL used in the paper's calculations.

A second occupational exposure study was conducted by Oestreich et al. (1997). The urinary levels of the hydroxylated

metabolite of epoxiconazole following dermal application compared to oral intake yielded an estimate of 1–2.5% dermal absorption using the assumption of 100% absorption from oral exposure. A field study yielded a very wide range of dermal exposure estimates: 60–10 000 µg/person/day as determined in 10 field applicators. The authors calculated that 1–100 µg/person/day would be absorbed from dermal exposure in the field. Inhalation exposure was negligible.

In the DAR (2006a) on epoxiconazole, the operator's systemic exposure was estimated to account for up to 48% of the AOEL of 0.01 mg/kg/d. By wearing gloves during mixing and loading, exposure would be about 2% of the AOEL although it is about 0.025 mg/kg/d in case no gloves are used. Exposure of bystanders was not expected to exceed the AOEL.

Human exposure from agricultural applications to consumers

There is little information in the peer-reviewed literature regarding what human exposure levels might be following normal registered agricultural applications, particularly with respect to typical consumers who might ingest plant sources which had received direct agricultural applications of epoxiconazole, or from animal sources which had fed upon plant sources which had received treatment with epoxiconazole.

Poulsen et al. (2009) performed a proficiency test that was to be used to verify analytical laboratory competence. Pesticide residues, including epoxiconazole, were quantified in cereals from both naturally incurred sources and from intentional spiking of samples. Epoxiconazole was applied to wheat fields at 1000 g/h, at 20–30 days before harvest. Following harvest the wheat was dried to contain no more than 15% mass fraction of water. The average residue of epoxiconazole on wheat, using the data from the laboratories deemed reliable in this proficiency test, was 0.176 mg/kg.

With respect to potential consumer exposures, the residue levels of epoxiconazole as assessed from field studies using practical conditions are below the limit of quantitation (0.05 mg/kg) in the edible parts of rice, spinach, potatoes, cucumbers, oilseed rape, soybeans, turnips, radishes and sugar beets which were cultivated as rotational crops following cereals European Food Safety Authority (EFSA, 2008). In beef or chicken tissues, the most abundant residue was the parent compound. Residues in animals that had eaten grains were no more than 0.1 mg/kg.

In the DAR (2006a) the theoretical maximum intake has been calculated using national and UK diets and the WHO European diet, which cover all categories of consumers including infants and toddlers, as well as high consumption patterns. All three calculations revealed exposures which are appreciably less than the ADI for epoxiconazole.

While potential residues of epoxiconazole in consumers have been shown to be relatively low, as indicated above, the fact that epoxiconazole is readily metabolized to excretable metabolites means that it will not bioaccumulate. The regulatory studies of absorption, distribution, metabolism and excretion (ADME) in laboratory rodents as reported in the DAR indicate that epoxiconazole is readily absorbed following oral administration and effectively excreted; 90–95% of an orally administered dose was excreted within 72 h. Based on urinary and biliary elimination, oral bioavailability was about 80% in males and 50% in females. Absorption through rat skin was appreciably lower, with less than 20% of an administered dose being absorbed by 72 h, with an overall dermal absorption rate of 3% being derived, with slower passage of the compound through human skin observed than through rat skin.

As described in the DAR (2006a) in mammals, Phase I biotransformation of epoxiconazole leads to hydrolytic opening of the oxirane ring, hydroxylation of the chlorophenyl ring and to a lesser extent of the fluorinated aromatic ring. In addition, cleavage of the carbon bridge between the two aromatic nuclei occurs. The most important Phase II reaction is formation of glutathione adducts at the chlorophenyl ring, substitution of aromatic chlorine and opening of the oxirane ring and formation of arene oxides. Further metabolism of the glutathione adducts occurs, with glucuronidation and sulfation of these products resulting in numerous other Phase II metabolites, isolated from the urine and the bile. Collectively, the tissue distribution and pharmacokinetic experiments indicated that epoxiconazole is unlikely to accumulate in tissues.

In summary, the likelihood of exposure of humans to epoxiconazole from its approved agricultural use is low, because the residues in edible parts of treated crops are low, frequently below the detection limits. Additionally, because of the ready metabolism, epoxiconazole is unlikely to bioaccumulate in food animals (as reflected by the low residues in edible parts of food animals) or in humans.

Risk assessment for epoxiconazole and for related azole fungicides

The risk assessment paradigm incorporates the hazard (i.e. toxicity) information and the exposure information for the target compound in order to calculate a risk (i.e. probability of harm at a given level of exposure). For a human health risk assessment, the hazard data most frequently comes from the guideline studies conducted on laboratory animals that are provided to the regulatory agency. The exposure data are frequently estimates based on residues of foods (if this is a food use pesticide) and food consumption data. The goal is to compare likely exposure levels to observed toxicity levels (usually in laboratory animals) to determine what level of safety may be present for the compound under its current or proposed uses. The risk assessment process usually incorporates safety factors/uncertainty factors to provide larger margins of safety between exposure levels and toxicity levels. In this section we attempt to compare the food residues of epoxiconazole to the levels of epoxiconazole that cause toxicity in laboratory animals to determine the margins of safety when epoxiconazole is applied to crops according to its approved agricultural usage. We also compare the exposure levels of epoxiconazole from food residues to the levels of human exposure occurring with the medical use of some of the therapeutic anti-fungal azoles because of the commonality of some of the mechanisms of action.

The residue level observed in wheat treated in the field once using 1000 g/ha epoxiconazole application from a published study was 0.176 mg/kg (Poulsen et al., 2009). It should be noted that this application level is 8 times higher than the recommended application rate of 125 g/ha (although this application rate can be applied twice per year, and therefore this application level would be 4 times higher than the recommended application rate). One fourth of the 0.176 mg/kg measured in this study is 0.044 mg/kg, which compared favorably with the residue levels cited in the DAR (2006a), below. Information on crop residues comes from BASF studies described in the DAR (2006a). Most of the residues of edible parts of crops were near or below the Limit of Quantitation (LOQ) of 0.05 mg/kg, with the Supervised Trials Median Residue (STMR) values for wheat grains, barley/oat grains, and sugar beet roots at 0.05, 0.06 and 0.05 mg/kg, respectively; the highest residues reported in these trials were 0.1, 0.39 and 0.05 mg/kg, respectively. The proposed Maximum Residue Levels (MRLs) for these three commodities are 0.2, 1.0 and 0.05 mg/kg, respectively. Most of the animal products assayed were at or below the LOQ. Proposed MRL's for beef, chicken, pork, milk and eggs are 0.01 mg/kg.

EFSA (2012) set an ADI of 0.008 mg/kg/d, which is derived from the NOAEL of the 18-month carcinogenicity study in mice (0.8 mg/kg/d) and incorporated a safety factor of 100.The Acute Reference Dose (ARfD) was set at 0.023 mg/kg based on the NOAEL obtained in the two generation reproduction study in rats (2.3 mg/kg/d) applying a safety factor of 100.Therefore for a 60 kg person the ADI would be 0.48 mg/d and the ARfD would be 1.38 mg, and it is unlikely that a person would ingest sufficient food to approach the ADI or the ARfD when the maximum allowable residues for various plants are 0.05-1.0 mg/kg commodity. The calculations in the DAR (2006a) indicate exposure from consumption of epoxiconazole contaminated commodities would be under the ADI. It is noteworthy that a recent report on probabilistic cumulative risk analysis (using reproductive/developmental endpoints different than those emphasized in the present paper) on four agricultural azole fungicides, including epoxiconazole, concluded that even cumulatively, dietary exposure to these four azoles is unlikely to pose an acute risk to consumers; the study used azole residues on foods reported in Denmark or Sweden for their exposure assessment (Jensen et al., 2013).

The exposure levels of therapeutic azole antifungals are substantially higher than projected exposure levels for agricultural azole pesticides occurring as residues on commodities (Figure 5). As mentioned earlier, few reports exist that suggest adverse consequences to the fetus of maternal use of therapeutic azoles to cure vulvovaginal fungal infections, and some of the datasets involved large numbers (tens of thousands) of individuals. To summarize, several reports showed no evidence of teratogenic effects for econazole, miconazole, clotrimazole and ketoconazole at the therapeutic dosages in the range of 100–200 mg/d for 3–10 days (Czeizel et al., 1999, 2003, 2004b; Kazy et al., 2005a). The reports that provided statistics suggestive of teratogenic

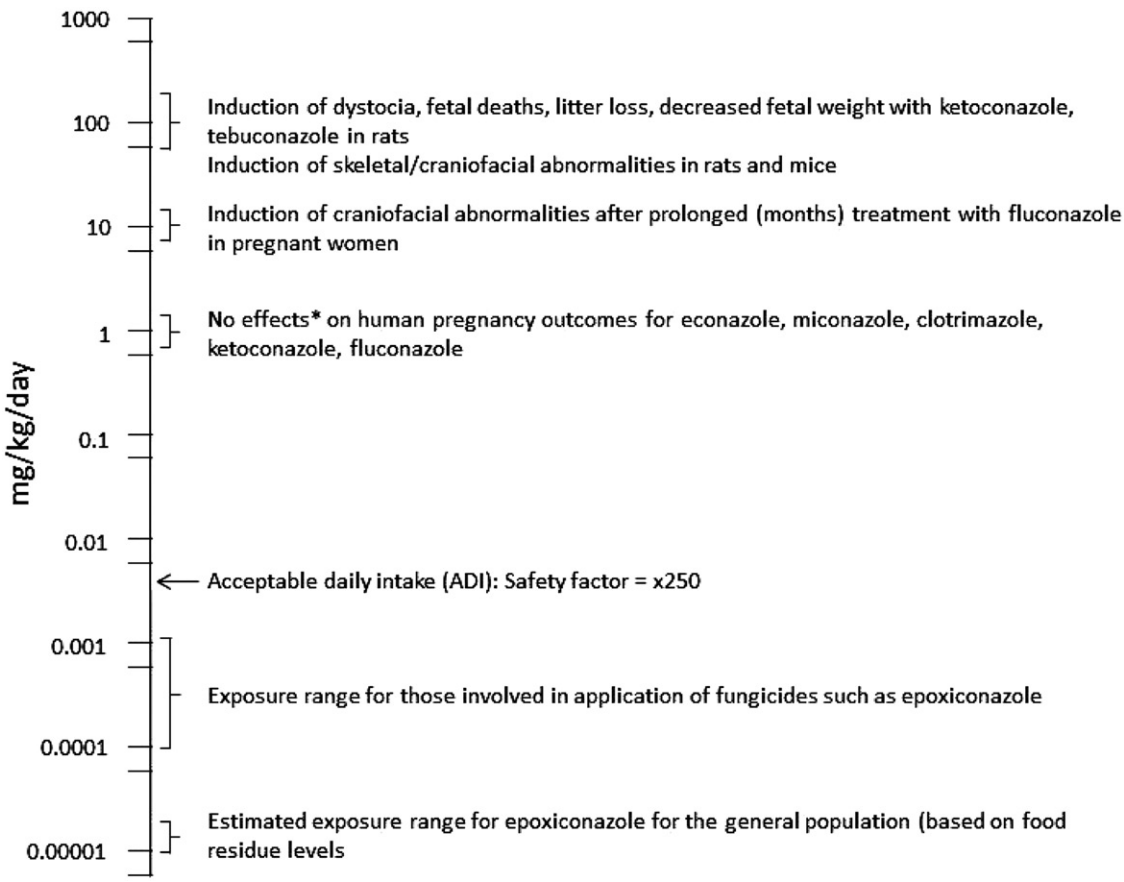

Figure 5. Estimated levels of human exposure for epoxiconazole in perspective to the human and experimental animal pregnancy exposure to azole compounds and effects. The summary data, and ranges, are derived from numerous studies that are cited and explained in the text.

effects indicated: metronidazole alone or together with miconazole, and fluconazole at dosage levels of 100–800 mg/d for 10 days to months, but the number of cases were small and the results may have been subject to recall bias (Czeizel & Rockenbauer, 1998; Kazy et al., 2005b,c; Pursley et al., 1996) These studies involved daily exposure to 100's of mg of the therapeutic azoles, which are 2–3 orders of magnitude above the epoxiconazole regulatory limits of 0.48 mg/day for the ADI (Figure 5) and 1.38 mg/d for the ARfD (calculated for a 60 kg adult from the ADI of 0.008 mg/kg/d and the ARfD of 0.023 mg/kg/d derived from the rodent studies). It is recognized, of course, that this comparison is between different azoles, and, as indicated previously, there are likely to be differences in potency and/or disposition.

The dosages of fluconazole that caused severe craniofacial and other defects were 400 and 800 mg/d and are about 833 and 1667 fold higher than the ADI (Figure 5) and 290 and 589 fold higher than the ARfD (calculated for a 60 kg adult) for epoxiconazole.

Recognizing that there would be differences in potency and/or disposition, with the safety factors built into the regulatory limits, such as an ADI, there is certainly a large margin of safety between estimated levels of exposure to epoxiconazole from residues resulting from standard agricultural applications and levels of azole antifungals which have resulted in adverse pregnancy outcomes in humans.

Conclusion

In conclusion, we recognize, of course, that there are always numerous uncertainties inherent in any risk assessment because of the interspecies extrapolations, the estimation required for exposure assessment and the use, on occasion, of surrogate measures when the needed information is not available, such as when NOAEL's are not available. In this case, we conclude that the exposure levels of humans to epoxiconazole from its approved agricultural use would be very far below the levels that would cause any toxic effect, especially any endocrine disruptive effect, based on the animal toxicity studies. The only human toxicity data that our literature search uncovered was for the therapeutic antifungal azoles, which are likely to have similar mechanisms of toxicity as epoxiconazole did in animal tests. These human toxic effects were only apparent at very high repeated daily doses (as seen in anecdotal human case reports) or they represent only a very few cases in epidemiological studies that were deemed susceptible to recall bias. The vast majority of exposures of women to therapeutic azoles did not result in adverse pregnancy outcomes.

Mechanistic studies have indicated that azoles are inhibitors of aromatase (CYP19), which can lead to adverse pregnancy outcomes. However, of the two types of effects sometimes observed in laboratory rodent tests, fetal resorption and cleft palate, only the former is likely to result from endocrine disruption resulting from CYP19 inhibition, and the differences between humans and rodents in the main estradiol production site and the level of dependency of the pregnancy on estradiol suggest that some of the adverse effects observed in rodents are not relevant to humans. Cleft palate is likely to result from mechanisms distinct from CYP19 inhibition, such as alterations in retinoic acid levels. Therefore we conclude that exposure to epoxiconazole as food residues would be without harm to people and without harm to the developing fetus, and would not elicit effects through an endocrine disruptive mechanism.

Wildlife

In considering the effects of epoxiconazole on wildlife species we have considered the responses by taxonomic group. Where possible we focus on the effects related to epoxiconazole and azole fungicide exposures and build towards an exposure and risk assessment for the grouping.

Mammalian wildlife

Effects assessment for epoxiconazole

There are limited data concerning the risks to wild mammals following the agricultural use of epoxiconazole. The concern is that wild mammals may be exposed to residues of the active substance directly or by the consumption of contaminated feed items. The hazard assessment for epoxicionazole draws heavily from laboratory rat and/or mice studies. For the rat the acute oral toxicity in terms of LD_{50} was 3160 mg/kg (Figure 6) and for the two-generation reproduction toxicity test a NOAEL was 2.3 mg/kg/d (DAR, 2006a). The most sensitive endpoint in rats and mice was liver toxicity.

Exposure and risk assessment for mammalian wildlife

The risk assessment for wild mammals performed according to European Union Guidelines relies on the calculation of a toxicity exposure ratio (TER). The TER is calculated as the toxicity measure such as the LD_{50} divided by the estimated theoretical exposure (ETE mg/kg/d). This equates to the daily dietary dose (DDD) which is defined by the food intake rate of the species of concern, the body weight of the species of concern, the concentration of a substance in/on fresh diet and the fraction of diet obtained in the treated area. A TER $\geq$ to 10 for short term studies or $\geq$ to 5 for long term studies indicates no additional refinement needed and little risk from exposure of an indicator species to the pesticide of interest. Conversely, a TER of less than the short term and long term thresholds would necessitate proceeding to a more refined exposure assessment as this would indicate some level of concern and/or risk of exposure to the pesticide (EFSA, 2009).

For the initial assessment the ETE assumes that the contaminated diet is not avoided (AV = 1), the animals satisfy their entire food demand in the treated area (PT = 1), the animals feed on a single food type (PD = 1) and concentration in the fresh diet is comparable to the maximum application rate. For cereals, the calculated ETE was between 0.40 and 29.6 mg/kg. Under the highly conservative assumptions of a Tier 1 risk assessment, the TER (Thune et al., 2005) for herbivorous and insectivorous mammals were higher than the trigger set by European Union Guidelines indicating little risk. The TER (long term) was 0.27 for small herbivorous mammals, which was less than the threshold of 5, thus triggering a revised assessment.

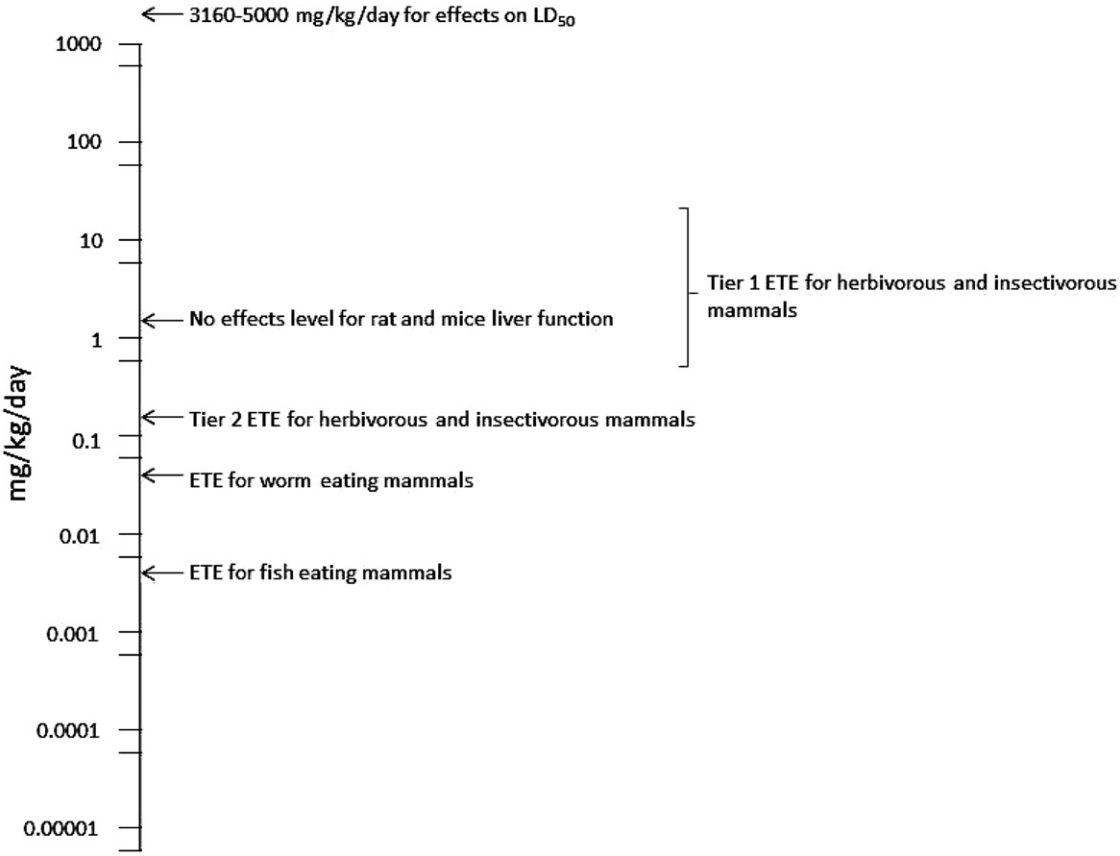

Figure 6. Relationship between exposure concentrations and biological effects of epoxiconazole in mammals.

In refining the exposure assessment, additional parameters were considered. This included: measured epoxiconazole residues the time weighted average for epoxiconazole decline in the environment, the concentration present after the last application compared to a single application, and proportion of the diet and times spent in treated-area by the species of concern. Taking these factors into account, the ETE was reduced to about 0.15 mg/kg. The Tier 2 long-term assessments for herbivorous mammals (hare, wood mouse and vole) result in a TER (long term) which are higher than the trigger of 5 set by European Union Guidelines which indicated little risk.

Other studies considered the risks to fish eating and worm eating mammals. In this case the ETE was 0.0046 mg/kg and 0.038 mg/kg for fish eating mammals and worm eating mammals, respectively. The corresponding TERs were in excess of the threshold of 5 and support the conclusion that the application of epoxiconazole in cereals does not give rise for concern with respect to accumulation of epoxiconazole in the food chain or for concern of secondary poisoning.

Birds

Toxicity tests with epoxiconazole

A number of studies have assessed the effects of epoxiconazole and two of its degradation products, triazolyl acetic acid and triazolyl alanine, in birds. The primary species for these studies include the bobwhite quail (*Colinus virginianus*), Japanese quail (*Coturnix coturnix japonica*), and mallard duck (*Anas platyrhynchos*).

Acute toxicity tests with epoxiconazole in the bobwhite quail revealed some modest effects on body weight, but the acute oral toxicity of epoxiconazole was low with the LD50 at $\geq$2000 mg/kg body weight (DAR, 2006b). Similarly in short term dietary toxicity studies with the bobwhite quail and mallard duck, the LC50 was $\geq$5000 mg/kg of diet (DAR, 2006b). There was no indication that the plant specific metabolites triazolyl acetic acid and triazolyl alanine exhibit increased toxicity to birds (Figure 7).

In terms of reproductive effects in the mallard duck, epoxiconazole at 500 ppm caused no statistically significant effects on a series of reproductive endpoints. There were modest reductions in the numbers of eggs laid, live 2 and 3 week embryos, normal hatchlings, and 14 day survivors. The NOEL for this study was 150 ppm. In studies with the bobwhite quail, epoxiconazole at 50 and 500 mg/kg caused a reduced but not statistically significant reduction in fertility rate, resulting in lower numbers of hatched chicks and 14-day old surviving chicks. The NOEL was 10 mg/kg. In studies with the Japanese quail all reproductive parameters evaluated according to the OECD guideline were not statistically significant or dose related up to the highest concentration tested of 500 ppm. Testes weight was not affected and there were no impacts on hormone levels, fertility and reproductive outcome in terms of laying rate or percentage of fertile eggs. Likewise, treatment had no influence on the eggs or chick parameters evaluated. However, histological evaluations revealed decreased spermatogenesis at 50 and 500 ppm (6.6 and 66 mg/kg/d) and this effect was not seen in controls or animals receiving 10 ppm (1.23 mg/kg/d) of epoxiconazole.

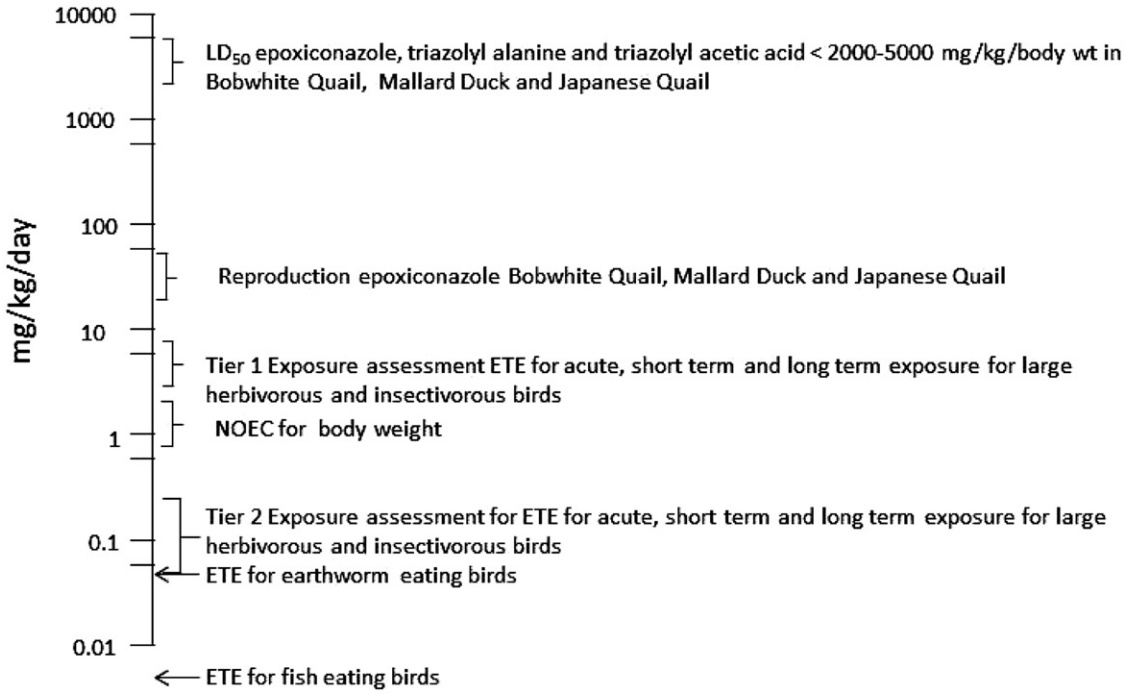

Figure 7. Relationship between exposure concentrations and biological effects of epoxiconazole in birds.

There was a statistically significant reduction in spermatid numbers and testicular canniculi with visible germ cells at 50 and 500 ppm. Accordingly the NOEL was taken as 10 ppm (Grote et al., 2008). In terms of risk assessment, the NOEC of 1.0 mg/kg was established based not on reproductive endpoints but rather on reductions in body weight.

Effects of other azoles on endocrine function in birds

There is a considerable literature on the effects of azole compounds other than epoxiconazole on various endocrine and reproductive endpoints in avian species. Many of these studies were pharmacological in nature and were designed to ask questions that explored the mechanisms and pathways that control development or reproductive function in avian species. For example, several studies showed that treatment of chicken (*Gallus gallus domesticus*) and turkey (*Meleagris gallopavo*) embryos with fadrozole caused genetic females to be sex reversed Studies in both embryonic (Abinawanto et al., 1997; Albrecht et al., 2000; Vaillant et al., 2001b, 2003) and mature laying chickens (Nishikimi et al., 2000; Sechman et al., 2003) have confirmed that fadrozole acts by decreasing aromatase activity and or expression. In the case of mature chickens, twice daily injections of fadrozole at 0.1 mg/kg body weight led to significant reductions in 17β-estradiol and increased testosterone levels and delayed oviposition times. Other studies with canaries (*Serinus canaria*) showed that 10 daily injections of fadrozole at 2 mg/kg/d delayed the emergence of copulation solicitation displays in response to conspecific songs and reduced egg-laying, and prevented incubation (Leboucher et al., 1998). In other studies, treatment of non-breeding, male song sparrows (*Melospiza melodia*) with fadrozole through a mini-osmotic pump at a concentration of 12 mg/kg for 14 days, reduced both neural aromatase activity and aggression relative to controls (Soma et al., 2004a,b) and led to a qualitative increase in overall testes size (Soma et al., 2000a,b)

Exposure assessment: epoxiconazole

Owing to the varied diets consumed by different species of birds (green plant material, seeds or prey such as insects, earthworms and fish), it was necessary to consider exposure to a variety of avian species. These data are covered in detail in the DAR (2006b) and selected data are summarized in Table 1. For the Tier 1 exposure assessment, ETEs of between 2.72 and 9.37 mg/kg were calculated for large herbivorous birds and insectivorous birds based on the standard agricultural practice of a maximal single application rate of 0.125 kg epoxiconazole/ha. As the first tier risk assessments were near the threshold, a Tier II exposure assessment taking into consideration the additional information described above was required. With these refinements, ETEs of 0.04–0.24 mg/kg were calculated.

Other studies examining fish eating birds and earthworm eating birds necessitated an assessment of bioaccumulation and food chain transfer. As there is low bioconcentration of triazoles in fish, the risk of secondary poisoning of fish-eating birds via food chain magnification is considered to be low (Brock et al., 2011). Although the log K_{ow} of >3 suggests a potential for bioaccumulation, epoxiconazole is rapidly metabolized. Based on a maximum bioaccumulation factor (BCF) of 70 for rainbow trout (*Oncorhynchus mykiss*) the time weighted residue in fish of 35 µg/kg or a daily dose to the birds of 7 µg/kg. Food chain transfer of epoxiconazole may also occur in terrestrial food chains, from soil animals like earthworms to birds. However, earthworms show low epoxiconazole residues, arising primarily from ingested soil particles rather than from bioaccumulation (DAR, 2006b). Thus, the risk to mammals and birds through dietary exposure

Table 1. Summary of epoxiconazole exposure and risk assessments in birds.

Group	Daily dose mg/kg/d	Endpoint	Toxicity oral mg/kg/d	TER
Large herbivorous birds (goose; Tier 1)	5.14	LC50 short term NOAEL	>907	>176
	2.72		1.0	0.37
Large herbivorous birds (goose; Tier 2)	0.15–0.24	NOAEL	1.0	4.2–6.7
Insect-eating birds (wren; Tier 1)	3.77	LC50 short term	>907	241
	3.77	NOAEL	1.0	0.27
Insect-eating birds (wren; Tier 2)	0.04	LC50 short term NOAEL	1.0	25
Fish eating birds (heron)	0.007	LC50 short term	>907	129 571
		NOAEL	1.0	143
Earthworm eating birds (song thrush)	0.045	LC50 short term	>907	>20 156
		NOAEL	1.0	22

appears to be negligible. In the case of earthworm eating birds such as the song thrush (*Turdus philomelos*) and assuming that its diet consisted entirely of contaminated earthworms, a daily dose of 45 µg/kg was calculated (DAR, 2006b).

Risk assessment

It was concluded that the application of epoxiconazole according to approved agricultural practices will pose limited risk to birds under natural conditions (DAR, 2006b). In the initial assessment for large herbivorous birds and insectivorous birds, the TERs for acute and short term exposures were 200 or more and as such were considerably higher than the trigger of 10 assigned by the European Union Guidelines. The TER for long term exposure was 0.27–0.37 for large herbivorous birds and insectivorous birds, necessitating a refined assessment. Using the revised ETEs of 0.04–0.24 mg/kg, the estimated TER for long term exposures for geese, grey partridge (*Perdix perdix*), yellow hammer (*Miliaria citronella*) and marsh warbler (*Acrocephalus palustris*), ranged from 4.2 to 25 with the goose and grey partridge being near the threshold. It was further estimated that because the exposure assessment included a limited window of time when there would be young cereal shoots that could be consumed by these birds, the TER was considered as being acceptable because of probable low epoxiconazole exposure.

The calculated TER for a fish eating bird (heron) based on the LC50 from short term dietary studies and the lowest NOEL from reproduction studies, (>129 571 and 143, respectively) indicating a low dietary risk to fish eating birds. Similarly, the risk to earthworm eating birds considered the song thrush and assumed that its diet consisted entirely of contaminated earthworms. Based on the LC50 from short term dietary studies and the lowest NOEL from long terms studies the TER were >20 156 and 22 which were indicative of the low risk to earthworm eating birds.

Reptiles

There are no published data considering the risk assessment of epoxiconazole in reptiles. However, there are several studies showing that azoles affect sex determination and sexual differentiation in reptiles. Fadrozole and letrozole at concentrations of 1.8 µg/g of egg or higher induced the male phenotype in the parthenogenetic whiptail lizard (*Cnemidophohorus uniparens*) which normally develops as a female (Wibbels & Crews, 1994; Wennstrom & Crews, 1995). Other studies with turtles, including *Chelydra serpentina* and *Emys orbicularis*, and the alligator *Alligator mississippiensis* showed that fadrozole and/or letrozole promoted the development of males (Belaid et al., 2001; Crews & Bergeron, 1994; Dorizzi et al., 1994; Lance & Bogart, 1992; Rhen & Lang, 1994; Wibbels & Crews, 1994). These findings are consistent with the premise that azoles act by blocking aromatase and thereby increase the levels of androgens. Evidence in support of this mechanism in reptiles comes from studies showing that the lizard, *Podar cissicula*, treated with letrozole at 5 mg/g body weight for 30 days had elevated plasma testosterone levels and reduced 17β-estradiol levels (Cardone et al., 2002). Other studies showed that fadrozole at 2 or 10 µg in 10 µl of ethanol administered to the egg shell induced masculinization of the gonads in the turtle *Emys orbicularis* and in some individuals reduced aromatase activity to the levels seen in males (Richard-Mercier et al., 1995). Collectively, these studies suggest that reptiles do not appear to be uniquely sensitive to the azoles compared to other vertebrates. Nonetheless, both hazard assessment and exposure assessment related to the practical agricultural application of epoxiconazole remains as a data gap.

Amphibians

There are no published studies, of which we are aware, considering the reproductive or endocrine responses of amphibians to epoxiconazole. Studies examining the steroidogenic responses of the brain of the green frog (*Rana esculenta*) showed that the addition of 10 µM ketoconazole decreased the conversion of [^{3}H]-pregnenolone into C_{21} 17-hydroxysteroids and C_{19} ketosteroids (Do Rego et al., 2007). In the same studies addition of graded concentrations of ketoconazole induced a dose-dependent inhibition of the biosynthesis of 17-hydroxypregnenolone, 17-hydroxyprogesterone, dehydroepiandrosterone and androstenedione. Azoles affect sex differentiation in amphibians. For example, fadrozole added to the water at 300 µg/L or higher induced masculinization of females and decreased ovarian aromatase activity in the newt *Pleurodeles waltl* (Chardard & Dournon, 1999). Other studies have shown reductions in brain aromatase activity in the frog *Silurana tropicalis* following developmental exposure to fadrozole or clotrimazole (Berg et al., 2009; Langlois et al., 2010, 2011). These studies have shown that azole fungicides have a similar spectrum of activity in amphibians as in other vertebrates and that there are no obvious differences in relative sensitivity of this vertebrate class to these compounds. In addition, a recent

Table 2. Acute toxicity of Epoxiconazole to various species of fish based on 96 hr toxicity tests.

Species	Test Compound	LC50 mg/L	NOEC mg/L
Rainbow trout	Epoxiconazole	3.14	2.15 mg/L
Blue gill sunfish	Epoxiconazole	5.62	3.16
Common carp	Epoxiconazole	8.27	2.74
Rainbow trout	1,2,4 triazole	760	100
Rainbow trout	BAS 480 13F	0.059	0.025

review by Weltje et al. (2013) revealed that fish and amphibian toxicity data for various toxic substances, including many plant protection products, are highly correlated and that fish are generally more sensitive than amphibians. Therefore, the relatively low toxicity of epoxiconazole to fish (see below) would seem to be applicable to amphibians.

Several studies have reported teratogenic effects of azole fungicides in amphibians. For example, exposure of the African clawed frog (*Xenopus laevis*) to triadimefon (2.7 µM and higher) or triadimenol (250 µM and higher) affected development of the branchial apparatus during neurulation (Groppelli et al., 2005; Menegola et al., 2006; Papis et al., 2006). These malformations resemble those seen following exposure to all-trans-retinoic acid (Menegola et al., 2006) and involves increased expression of Cyp 26A (Di Renzo et al., 2011; Papis et al., 2007). Citral, a retinoic acid inhibitor, reduced the teratogenic effects of the fungicide supporting the notion that endogenous RA is involved in the induction of the branchial abnormalities. Given the similarities with what was reported in mammals for cleft palate, these data suggest a high degree of conservation of the pathways controlling cranial development. In any event, the concentrations of the azoles required to induce such effects were much higher than the levels of fungicides seen in the environment.

Fish

Effects assessment: studies with epoxiconazole

A series of studies have considered the acute, chronic and lifecycle effects of epoxiconazole and some of its degradation products in fish. Epoxiconazole has low toxicity to fish based on 96 h acute toxicity tests. The LC50 value for various fish species ranged from 3.14–8.27 mg/L (Table 2). The metabolite 1,2,4-triazole was about 2 orders of magnitude less toxic than the parent compound.

Studies examining the long term effects of epoxiconazole in fish have included a 28 day juvenile growth test, 96 day early life stage study and 3 fish full lifecycle tests. In the 28 day tests, the NOEC for epoxiconazole was 0.01 mg/L and the most sensitive endpoints were body weight and length. The NOAEL was 0.017 mg/L in the early lifecycle tests. The no observed ecologically adverse effect concentration (NOEAEC) in lifecycle tests with the zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) were 0.012 mg/L and 0.010 mg/L, respectively. The lowest NOEC resulting from fish studies was 0.003 mg/L and was based on a transient growth reduction at certain life stages. The lowest NOEAEC was 0.01 mg/L based again on growth effects and on potential for endocrine effects (influence on sex development of fish exposed during sensitive stages).

Effects of azoles on endocrine and reproductive endpoints.

A large number of studies have examined the effects of azoles on endocrine and reproductive responses in fish. This has included effects and various responses related directly to estrogen synthesis and actions. Some studies have examined effects on aromatase expression and/or activity, or on vitellogenin production, or on androgen levels and steroidogenesis. Other studies have concentrated on apical endpoints such as sexual differentiation or spawning success. Very few of these studies have considered the actions of epoxiconazole but have utilized prochloraz or ketonconazole which were tested because of their inhibitory effects on various P450s.

Effects on vitellogenin. Numerous studies have examined the effects of azole fungicides on the vitellogenin response in fish (Table 3). In 17 of 18 experiments testing the effects of azoles in females, the levels of vitellogenin mRNA or protein were decreased. This is consistent with the inhibitory effects of azoles on aromatase and 17β-estradiol production which are required for vitellogenin synthesis. Much of the research has been done using prochloraz for which LOEC values range from 20 to 284 µg/L for studies with the fathead minnow to 48–202 µg/L for studies with the zebrafish. The response to these azoles varies not only between labs (Holbech et al., 2012) but also with stage of development. The vitellogenin response in males was far more variable. Work in zebrafish testing prochloraz (Kinnberg et al., 2007) and fathead minnow testing fadrozole have reported increased vitellogenin levels following exposure. Yet other studies have reported reductions in vitellogenin levels or no effects at high concentrations of test compound (Table 3). The mechanism by which the azoles increase vitellogenin levels in males is not known (Panter et al., 2004). Collectively the effects on vitellogenin were seen at concentrations that are much higher than the concentrations of epoxiconazole that affect the most sensitive toxicological endpoints such as growth.

Effects on aromatase. Numerous studies have examined the effects of azoles on aromatase activity in fish (see Table 4). In the majority of these studies, fadrozole or prochloraz decreased the expression and or activity of aromatase and these effects were typically seen at high concentrations of the azole. Paradoxically several studies have shown that fish will exhibit increased brain or ovarian aromatase expression following exposure to azoles. Most often these responses were seen in short term exposure scenarios and are thought to reflect the ability of the fish to mount a compensatory response to the direct, competitive inhibition of aromatase activity (Villeneuve et al., 2006).

Effects on steroidogenesis. A variety of *in vivo*, *in vitro* and *ex vivo* experiments have been used to determine the effects of azoles on steroidogenesis. In 21 day tests with fathead minnows, ketoconazole at the highest concentration increased the gonadosomatic index (GSI) in females but had no effect on plasma vitellogenin, 17β-estradiol or testosterone levels (Villeneuve et al., 2007b). Similarly in males, ketoconazole increased GSI but again had no effect on plasma vitellogenin, testosterone or 17β-estradiol levels. Histology revealed no

Table 3. Summary of the effects of azole fungicides on the vitellogenin response in fish.

Compound	Species	Test	Sex	Response	LOEC	Reference
Prochloraz	Fathead minnow	Post hatch-60d	F	↓ VTG	29 µg/L	Holbech et al. (2012)
Prochloraz	Fathead minnow	Post hatch-120d	F	↓ VTG	106 µg/L	Holbech et al. (2012)
Prochloraz	Fathead minnow	Post hatch-60d	M	↓ VTG	>284 µg/L	Holbech et al. (2012)
Prochloraz	Fathead minnow	Post hatch-120d	M	↓ VTG	301 µg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	F	↓ VTG	48 µg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	F	↓ VTG	99 µg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	F	↓ VTG	183 µg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	M	↓ VTG	>320 µg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	M	↓ VTG	44 µg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	M	↓ VTG	135 µg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	F	↓ VTG	202 µg/L	Kinnberg et al. (2007)
Prochloraz	Zebrafish	Post hatch-60d	M	↑ VTG	16 µg/L	Kinnberg et al. 2007
Prochloraz	Fathead minnow	Adult-21d	F	↓ VTG	20 µg/L	Jensen & Ankley (2006)
Fadrozole	Fathead minnow	Adult-21d	M	↓ VTG	100 µg/L	Jensen & Ankley (2006)
Prochloraz	Fathead minnow	Adult-21d	F	↓ VTG	116 µg/L	Ankley et al. (2005)
Prochloraz	Fathead minnow	Adult-21d	M	↓ VTG	>311 µg/L	Ankley et al. (2005)
Prochloraz	Fathead minnow	Adult-2d	F	↓ VTG	284 µg/L	Ankley et al. (2009a)
Fadrozole	Fathead minnow	Adult-21d	F	↓ VTG	1.4 µg/L	Ankley et al. (2006)
Fadrozole	Fathead minnow	Adult-21d	M	↓ VTG	57 µg/L	Ankley et al. (2006)
Fadrozole	Fathead minnow	Adult-21d	F	↓ VTG	24.8 µg/L	Panter et al. (2004)
Fadrozole	Fathead minnow	Adult-21d	M	↑ VTG	24.8 µg/L	Panter et al. (2004)
Fadrozole	Fathead minnow	Juvenile-14d	M/F	↓ VTG	87.7 µg/L	Zerulla et al. (2002)
Fadrozole	Zebrafish	Post hatch-20–60d	M/F	↑ VTG	10 µg/L	Andersen et al. (2004)
Ketoconazole	Fathead minnow	Adult-7d	F	↓ VTG	468 µg/L	Ankley et al. (2007)
Ketoconazole	Fathead minnow	Adult-21d	M	VTG	>357 µg/L	Ankley et al. (2007)
Ketoconazole	Fathead minnow	Adult-4d	F	↓VTG	311 µg/L	Ankley et al. (2012)
Prochloraz	Fathead minnow	Adult-1d	M	↓ VTG	>300 µg/L	Skolness et al. (2011)
Fadrozole	Fathead minnow	Adult-2d	F	↓ VTG	4.1 µg/L	Villeneuve et al. (2009)
Fadrozole	Fathead minnow	Adult-4d	M	↓ VTG	4.1 µg/L	Villeneuve et al. (2009)
Prochloraz	Medaka	Adult-7d	F	↓ liver VTG II expression	3 µg/L	Zhang et al. (2008)
Prochloraz	Medaka	Adult-7d	M	↑ liver VTG I expression	3 µg/L	Zhang et al. (2008)
Ketoconazole	Medaka	Adult-7d	F	↓ liver VTG II expression	3 µg/L	Zhang et al. (2008)
Ketoconazole	Medaka	Adult-7d	M	↓ liver VTG I expression	300 µg/L	Zhang et al. (2008)

remarkable effects in ovaries of ketoconazole-exposed fish but a marked proliferation of interstitial (Leydig) cells in testis of treated fish. These responses were not in agreement with ex vivo studies which showed that testosterone production by both ovarian and testicular tissue was decreased by exposure to ketoconazole. Surprisingly, several studies have shown that high levels of ketoconazole increased the expression mRNA transcripts for cytochrome P450 side-chain cleavage (Cyp11A) in males and females and cytochrome P450 c17α hydroxylase/17,20lyase (Cyp17) in males. In subsequent analysis, Villeneuve et al. (2007c) reported that exposure to ketoconazole at 400 µg/L increased the expression of Steroid Acute Regulatory Protein (StAR), cytochrome b5 and 20β-hydroxysteroid dehydrogenase (20βHSD) mRNA in the testis of fathead minnows. Collectively the suite of changes observed in these studies was interpreted as evidence of adaptive or compensatory responses of the fathead minnow to impaired steroidogenic capacity following azole exposure (Ankley et al., 2007; Villeneuve et al., 2007b).

In other studies, ovarian tissue from fathead minnows exposed to ketoconazole led to a significant reduction in testosterone and 17β-estradiol production after 8 and 12 h of incubation (Perkins et al., 2008). Expression of 11β- and 20β-hydroxysteroid dehydrogenases were down-regulated at 1 h and Cyp17 was down-regulated at 12 h, whereas aromatase was up-regulated after 6 h of incubation. Microarray analysis of the samples from the 12 h incubations showed that 2042 genes were differentially expressed with 1744 up-regulated while 298 were down-regulated at a cut off value of $p < 0.05$ (Perkins et al., 2008). Collectively these studies showed that ketoconazole had a broad range of actions on fathead minnow ovarian follicles.

In recent studies, sexually mature fathead minnows were exposed to waterborne ketoconazole for up to 8 days, following which animals were allowed to recover in clean water for up to 16 days (Ankley et al., 2012). Exposure to 30 and 300 µg/l ketoconazole led to a transient decrease in plasma testosterone levels in males on days 1 and 4 but by day 8 the levels in the 30 µg/L treated fish were higher than controls and in the higher dose group did not differ from controls. These results were generally consistent with the depression of testosterone production seen in *ex vivo* incubations of testicular tissue. Analysis of the expression of genes involved in steroid biosynthesis revealed a number of changes following exposure to ketoconazole. Expression of mRNA for Cyp11a and Cyp17 were up-regulated in the 30 and 300 µg/L treatments on days 1 and 8 of the exposure and, in the higher dose treatment, also on day 1 of the recovery. Expression of the two transcripts returned to control levels in both treatment groups during the latter portion of the recovery phase. Expression of StAR in the testis also was up-regulated by ketoconazole on days 8 and 1 of the exposure and recovery phases of the experiment, respectively. Transcripts of the FSH receptor also were significantly elevated 1 d after cessation of ketoconazole exposure. Plasma concentrations of 17β-estradiol in females showed little change following exposure to ketoconazole but there was a marked reduction in *ex vivo*

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Table 4. Summary of the effects of azole fungicides on aromatase in fish.

Compound	Species	Test	Sex	Response	LOEC	Reference
Prochloraz	Fathead minnow	Adult-21d	F	↓ brain activity	>311 µg/L	Ankley et al. (2005)
Prochloraz	Fathead minnow	Adult-21d	M	↓ brain activity	311 µg/L	Ankley et al. (2005)
Prochloraz	Fathead minnow	Adult-untreated	F	↓ brain and ovary activity	3767 µg/L	Ankley et al. (2005)
Fadrozole	Fathead minnow	Adult-21d	F	↓ brain activity	57 µg/L	Ankley et al. (2002)
Fadrozole	Fathead minnow	Adult-21d	M	↓ brain activity	57 µg/L	Ankley et al. (2002)
Fadrozole	Fathead minnow	Adult-untreated	F	↓ brain activity	158 µg/L	Villeneuve et al. (2006)
Fadrozole	Fathead minnow	Adult-untreated	F	↓ ovary activity	533 µg/L	Villeneuve et al.(2006)
Fadrozole	Fathead minnow	Adult-7d	F	↓ brain activity	16.1 µg/L	Villeneuve et al.(2006)
Fadrozole	Fathead minnow	Adult-7d	F	↑ ovary activity	1.85 µg/L	Villeneuve et al. (2006)
Fadrozole	Fathead minnow	Adult-7d	F	↓ brain CYP19B expression	5.5 µg/L	Villeneuve et al. (2006)
Fadrozole	Fathead minnow	Adult-7d	F	No effect brain CYP19A expression	>48.2 µg/L	Villeneuve et al. (2006)
Fadrozole	Fathead minnow	Adult-7d	F	↓ ovary CYP19B expression	1.85 µg/L	Villeneuve et al. (2006)
Fadrozole	Fathead minnow	Adult-7d	F	↑ ovary CYP19A expression	1.85 µg/L	Villeneuve et al. (2006)
Prochloraz	Fathead minnow	Adult-2d	F	↑ovarian cyp19a1a expression	311 µg/L	Ankley et al. (2009b)
Prochloraz	Medaka	Adult-7d	F	↓ brain cyp19b expression	300 µg/L	Zhang et al. (2008)
Prochloraz	Medaka	Adult-7d	M	↓ brain cyp19b expression	30 µg/L	Zhang et al. (2008)
Prochloraz	Medaka	Adult-7d	F	↑ ovary cyp19a expression	30 µg/L	Zhang et al. (2008)
Prochloraz	Medaka	Adult-7d	M	↑ testis cyp19a expression	3 µg/L	Zhang et al. (2008)
Ketoconazole	Medaka	Adult-7d	F	brain cyp19b expression	>300 µg/L	Zhang et al. (2008)
Ketoconazole	Medaka	Adult-7d	M	brain cyp19b expression	>300 µg/L	Zhang et al. (2008)
Ketoconazole	Medaka	Adult-7d	F	ovary cyp19a expression	>300 µg/L	Zhang et al. (2008)
Ketoconazole	Medaka	Adult-7d	M	↑ testis cyp19a expression	30 µg/L	Zhang et al. (2008)
Fadrozole	Zebrafish	Post hatch-35–70d	M/F	↓ testis cyp19a expression	500 µg/g diet	Fenske & Segner (2004)
Fadrozole	Zebrafish	Post hatch-35–70d	M/F	brain cyp19b expression	>500 µg/g diet	Fenske & Segner, (2004)
Fadrozole	Japanese flounder	30–100- d posthatch	All F	↓ gonad P450arom expression	100 µg/g diet	Kitano et al. (2000)
Ketoconazole	Fathead minnow	Adult-21d	F	No effectCyp 19 gene expression	>357 µg/L	Ankley et al. (2007)
Ketoconazole	Fathead minnow	Adult-*in vitro* ovary incubation	F	↑Cyp 19a gene expression	0.5 µM	Perkins et al. (2008)
Ketoconazole	Fathead minnow	Adult-8d	F	No effect Cyp 191a1 gene expression	>311 µg/L	Ankley et al. (2012)
Prochloraz	Fathead minnow	Adult- 6 h	M	↑Cyp 19a1a ovary gene expression	300 µg/L	Skolness et al. (2011)
Prochloraz	Fathead minnow	Adult-1d	M	No effect Cyp 19a1b ovary gene expression	>300 µg/L	Skolness et al. (2011)
Fadrozole	Fathead minnow	Adult-4d	F	↑VTG ovary CYP19A	4.1 µg/L	Villeneuve et al. (2009)

production of testosterone and 17β-estradiol production which did not return to control values until at least day 8 in the recovery period. Expression of Cyp11a and Cyp17 were both increased in a dose-dependent fashion on days 1 and 8 of the ketoconazole exposure, as well as on days 1 (Cyp11a) and 8 (Cyp17) of the recovery phase of the test. Collectively these studies have shown that ketoconazole affects steroidogenesis but that a number of compensatory mechanisms mediate these effects (Ankley et al., 2012).

In other studies, adult male zebrafish were exposed to measured concentrations in water of 71, 159 and 258μg/L of clotrimazole for 7 days (Baudiffier et al., 2012). Clotrimazole induced a concentration-dependent increase in gene expression of *Star*, *Cyp17a1*, and *Cyp11c1* gene expression and of Cyp17a1 and Cyp11c1 protein synthesis in Leydig cells, but androgen levels in blood remained unchanged. These studies demonstrate that azoles affect steroid synthesis but do so only at high concentrations.

There is evidence that prochloraz can also cause anti-androgenic effects in fish either through direct antagonism of the androgen receptor or through inhibition of Cyp17, a steroidogenic enzyme involved in testosterone production (Ankley et al., 2005, 2007). The available evidence suggests that different P450s may have varying sensitivities to different azoles. As an example, ketoconazole tends to be more effective in inhibiting Cyp11A/Cyp17 whereas fadrozole is more effective in blocking Cyp19 and prochloraz as blocking Cyp17 and Cyp19. These differences open questions as to the specificity of epoxiconazole and azoles in general.

Effects on other endocrine-dependent apical responses. Many studies have shown that azole fungicides affect sexual differentiation in fish (Table 5). Both waterborne and dietary routes of exposure increase the proportion of male fish. This finding is consistent with a reduction in aromatase activity thereby reducing estrogen availability, which is necessary for ovarian differentiation. The masculinizing effect of fadrozole was blocked by the co-administration of 17β-estradiol (Kitano et al., 2000). However these effects are seen at concentrations that are far in excess of environmentally relevant levels of epoxiconazole.

Several studies have shown that azoles negatively affect spawning success in fish (Table 6). In particular, fadrozole was far more effective than prochloraz or ketoconazole in reducing egg production in fathead minnows (Ankley et al., 2002, 2005, 2007). These studies have shown that azoles induce adverse effects at concentrations that greatly exceed the anticipated environmental levels of epoxiconazole. For example, spawning adult fathead minnows exposed to ketoconazole at 25 or 400 μg/L for 21 d led to a significant decrease in the number of eggs spawned and the number of spawning events (Ankley et al., 2007). In the same studies, ketoconazole at 100 μg/L had no effect on spawning success. Exposure to ketoconazole did not affect either fertility or hatch. In other studies, Zhang et al. (2008) examined the effects of prochloraz and ketoconazole on the spawning success and expression of genes along the hypothalamic-pituitary-gonadal (HPG) axis of the Japanese medaka (*Oryzias latipes*). Medaka exposed for seven days to 30 or 300 μg prochloraz/L or to 300 μg ketoconazole/L had significant reductions in the numbers of eggs that were spawned. These effects were associated with marked changes in the expression of a suite of genes in the gonads, livers and brains of these fish.

Prochloraz, epoxiconazole and imazalil strongly potentiated the gonadotropin-dependent induction of oocyte maturation of rainbow trout oocytes. Prochloraz and epoxiconazole alone also induced oocyte maturation (Monod et al., 2004). This is one of the few studies testing the actions of epoxiconazole in lower vertebrates.

Table 5. Summary of the effects of azole fungicides on sex ratio in fish.

Compound	Species	Test	Sex	Response	LOEC	Reference
Prochloraz	Fathead minnow	Post hatch-60d	M/F	↑ Males	284 μg/L	Holbech et al. (2012)
Prochloraz	Fathead minnow	Post hatch-120d	M/F	↑ Males	301 μg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	M/F	↑ Males	320 μg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	M/F	↑ Males	99 μg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	M/F	↑ Males	60 μg/L	Holbech et al. (2012)
Prochloraz	Zebrafish	Post hatch-60d	M/F	↑ Males	202 μg/L	Kinnberg et al. (2007)
Fadrozole	Zebrafish	Post hatch-14–40d	All F	↑ Males	10 μg/g diet	Uchida et al. (2004)
Fadrozole	Zebrafish	Post hatch-35–70d	M/F	↑ Males	500 μg/g diet	Fenske & Segner (2004)
Fadrozole	Chinook salmon	3 days post hatch-2 h exposure	All F	↑ Males	1000 μg/L	Piferrer et al. (1994)
Fadrozole	Japanese flounder	30–100 d post hatch	All F	↑ Males	1 μg/g diet	Kitano et al. (2000)
Fadrozole	Nile tilapia	7–37d post hatch	All F	↑ Males	40 μg/g diet	Kwon et al. (2000)
Fadrozole	Zebrafish	Post hatch-20–60d	M/F	↑undifferentiated and ↓females	10 μg/L	Andersen et al. (2004)

Table 6. Summary of the effects of azole fungicides on spawning (egg laying) in fish.

Compound	Species	Test	Sex	Response	LOEC	Reference
Prochloraz	Fathead minnow	Adult-21d	M/F	↓ egg production	116 μg/L	Ankley et al. (2005)
Fadrozole	Fathead minnow	Adult-21d	M/F	↓ egg production	1.4 μg/L	Ankley et al. (2002)
Ketoconazole	Fathead minnow	Adult-21d	M/F	↓ egg production	25 μg/L	Ankley et al. (2007)
Prochloraz	Medaka	Adult-7d	M	↓ egg production	30 μg/L	Zhang et al. (2008))
Ketoconazole	Medaka	Adult-7d	M	↓ egg production	300 μg/L	Zhang et al. (2008)

Recent studies have demonstrated the effects of azoles on early embryo development in the zebrafish. Hermsen et al. (2011) reported that flusilazole was the most potent triazole, followed by hexaconazole, cyproconazole, triadimefon, myclobutanil and triticonazole, in inducing developmental abnormalities. In other studies, difenoconazole at concentrations of 500 µg/L and higher contributed to hatching inhibition, abnormal spontaneous movement, slow heart rate, growth regression and morphological deformities (Mu et al., 2013). Domingues et al. (2013) showed that prochloraz at concentrations greater than 1.2 mg/L was also teratogenic in zebrafish embryos as they exhibited spine deformations, edema, and lack of body and eye pigmentation.

Exposure and risk assessment

Epoxiconazole can leach from soil into aquatic systems where it appears to be hydrolytically and photolytically stable and shows slow microbial degradation (EFSA, 2008). Within the aquatic environment, epoxiconazole tends to partition into the sediment which is consistent with its organic carbon adsorption coefficient (Koc) (DAR, 2006a). Biodegradation takes place, both in the water and the sediment phases, but at low rates. In natural water, 20% of the active substance was degraded after 15 days (period for 50% dissipation; DT_{50} is 52 days). Within the sediment, epoxiconazole is metabolized to 1,2,4-entriazole and BF480 entriazole. The DT_{50} in water for BF480 entriazole is 38–93 days and the DT_{90} in the whole system is 224-573 days. Overall, there is concern that aquatic organisms are exposed to epoxiconazole as it is regarded as being persistent in aquatic systems.

Predicted environmental concentrations in surface water (PECsw) (Figure 8) resulting from spray application of epoxiconazole (2 x 0.125 kg/ha) on cereal fields were calculated by the standardized worst case scenario, utilizing European surface water assessment of plant protection products. The worst case global maximum concentration was 0.837 µg/L for the drainage ditch scenarios and 1.215 µg/L for the stream runoff scenarios. Using the same modeling scenario, predicted worst case maximum sediment concentrations vary between 0.166 and 4.445 µg/kg (DAR, 2006a). Predicted actual concentrations in the water layer after 1 day vary from 0.001 µg/L under certain stream scenarios to over 0.033 µg/L in ponds to a maximum of 0.762 µg/L in ditches. Time weighted average exposure concentrations range from 0.032 µg/L in ponds to 0.834 µg/L in streams (DAR, 2006b). Corresponding sediment concentrations are 0.837 to 4.45 µg/kg. Alternative modeling by Probst et al. (2005) predicted in-stream concentrations of epoxiconazole which varied, depending on agricultural practices, between 2.9 and 91 µg/L as maximum values, and 0.19 and 6.2 µg/L as average values.

Measured environmental concentrations of epoxiconazole were reported by Berenzen et al. (2003). These authors analyzed river stretches up- and downstream to the effluents of five wastewater treatment plants, and found either no epoxiconazole or concentrations between 0.05 and 0.1 µg/L. Berenzen et al. (2005) used runoff-triggered samplers to monitor peak pesticide levels in streams situated in an agricultural area in Northern Germany, and found epoxiconazole concentrations up to a maximum of 2.7 µg/L; the measured values correlated significantly with predicted environmental concentrations. In conclusion, both prediction models as well as chemical-analytical studies indicate epoxiconazole concentrations in the aquatic environment which are in the low µg/L range. The bioconcentration of epoxiconazole in fish is low and was covered above.

Triazole fungicides reach the aquatic environment mainly through surface-runoff and spray drift but can also do so via wastewater effluents (Berenzen et al., 2003). Wastewater effluents are a source of triazoles, primarily in areas where wastewater treatment plants receive both domestic wastewater

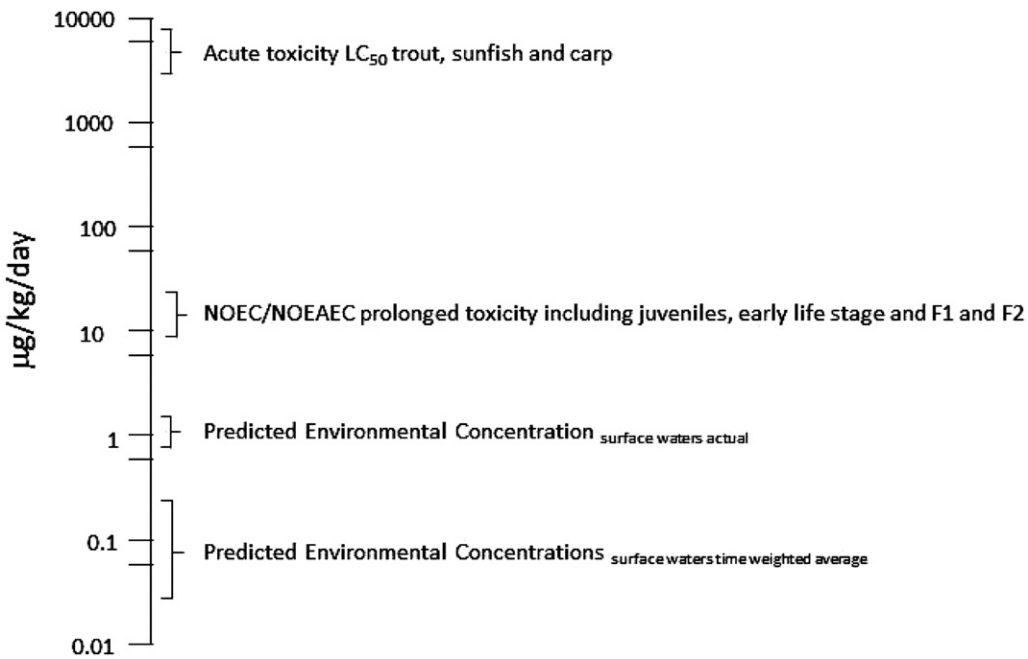

Figure 8. Relationship between exposure concentrations and biological effects of epoxiconazole in fish.

and rainwater from sealed surfaces. Following rainfall events, Berenzen et al. (2003) analyzed the effluents of five wastewater treatment plants from intensive agricultural areas and found epoxiconazole at concentrations from non-detectable up to a maximum of 0.2 µg/L. The contribution of the effluents to the river load of fungicides was found to be clearly lower than the contribution of surface run-offs.

The acute TER values for epoxiconazole in various waters for fish based on worst case assumptions meet the standard triggers considering spray applications in 1 meter distance to ditches, 1.5 meter distance to streams and 3.5 meter distance to ponds. If endpoints relevant for the population (NOEAEC greater than or equal to 0.01 mg/L) are used TER values for fish indicate a low risk due to chronic or endocrine effects (DAR, 2006b).

Invertebrates

Effects assessment for epoxiconazole

The available database on the toxicity of triazole fungicides in general and epoxiconazole in particular to invertebrates is limited. For example, the US EPA ECOTOX Database (http://cfpub.epa.gov/ecotox) contains only two reports on epoxiconazole toxicity to aquatic invertebrates and none for soil invertebrates.

In terms of studies with aquatic invertebrates, epoxiconazole was tested with crustaceans (*Daphnia magna*), which is a pelagic species, and with the arthropod, *Chironomus riparius*, which is a sediment-dwelling species. Acute toxicity, determined as 48-h-EC50 of epoxiconazole in *D. magna,* was 8.69 mg/L (nominal concentration), and the NOEC derived from the same test was 3.13 mg/L (DAR, 2006b). By comparison epoxiconazole affects non-target fungi including those relevant for the functioning of biofilms with NOEC values as low as 0.001 mg/L for some fungi (Dijksterhuis et al., 2011).

Chronic toxicity of epoxiconazole to invertebrates was assessed in 21-d-reproduction tests with *D. magna*, and the concentrations causing effects were about an order of magnitude lower than the concentrations causing acute toxicity. Both in the acute and chronic toxicity test with *D. magna*, the toxicity of the formulation was found to be higher than to be expected from the active substance content, e.g. the 21-d-NOEC was 0.08 mg/L in the formulation compared to 0.63 mg/L pure substance (Figure 9; DAR, 2006b). In *C. riparius*, emergence of the sediment-dwelling larvae was not adversely affected by a 21-d-exposure to epoxiconazole concentrations up to 0.0625 mg/L (DAR, 2006b). For *in situ* studies with the oyster, *Crassostrea gigas,* exposure to 0.01 mg/L epoxiconazole for 13 days had no effect on larval growth (Stachowski-Haberkorn et al., 2008). With respect to terrestrial arthropods, acute (48 h) oral and contact toxicity of epoxiconazole and the fungicide formulation (BAS 480 27F, 125 g/L) were tested in honey bees, *Apis mellifera* (DAR, 2006b).A concentration range of 6.25 to 100 µg/bee was tested, and LD_{50} values of acute oral toxicity were >83 µg/bee for the pure substance, and >69.9 µg/bee for the formulation. With respect to contact toxicity of epoxiconazole to bees, acute LD_{50} values were >100 µg/bee for the pure substance, and >59.7 µg/bee for the formulation. Thermoregulation of honey bees was found to be affected by doses of 0.85 µg/bee and higher (Vandame & Belzunces, 1998). For terrestrial arthropods other than bees, toxicity data are available for the fungicide formulation, BAS 480 27F. In the mite, *Typhlodomus pyri*, a 7-day laboratory administration led to a dose-dependent increase of mortality,

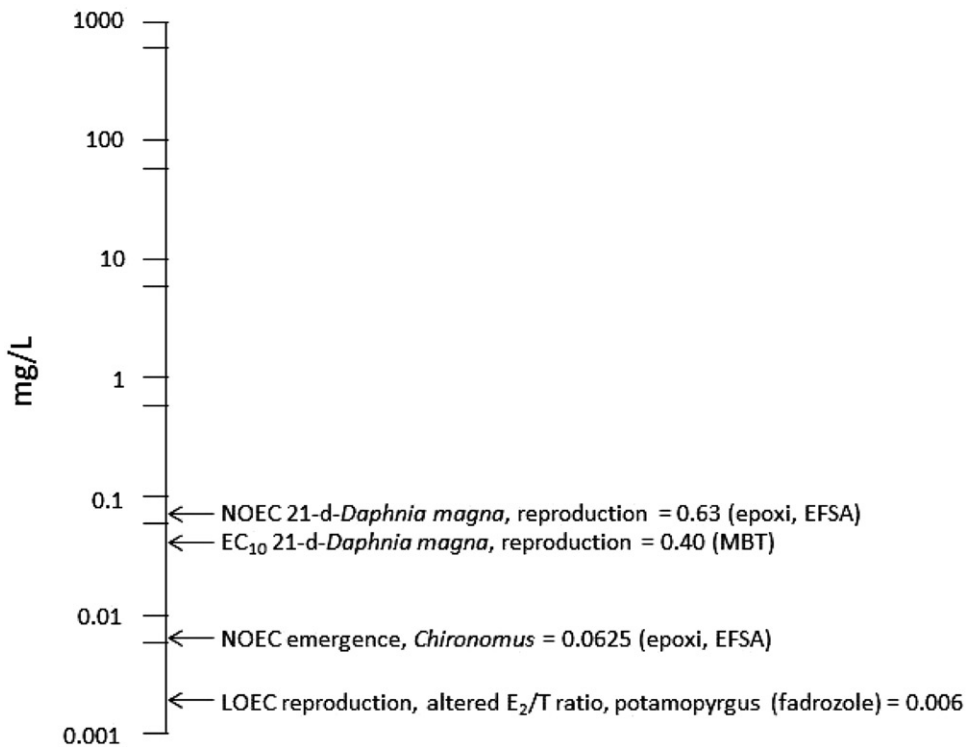

Figure 9. Effect concentrations of triazoles in invertebrates that might be mediated via endocrine mechanisms/P450 inhibition.

with mortalities between 10 and 82.8% for formulation application rates from 0.7 to 3.5 L/ha. Reproduction of the mites (measured after 14-day exposure) showed no clear dose dependency, with decreases of 31.7 to 44.4% for application rates between 0.7 and 1.5 L/ha. The parasitic wasp *Aphidius rhopalopsiphi* showed 40.8% mortality after 48-h exposure to 2 L formulation/ha (250 g/ha), yet reproduction was not affected at this concentration. The Ladybird, *Coccinella septempuncta*, showed no significant increase of mortality or a significant decrease of reproduction after a 14-d exposure to 1.5 L/ha (187.5 g/ha) (concentrations other than 1.5 L/ha were not tested). The same application rate (1.5 L/ha) resulted in 3% mortality of the ground beetle, *Poecilus cupreus*, after a 14-day exposure period; this treatment was not associated with obvious signs of sublethal damage nor did it reduce feed consumption of the beetles (concentrations other than 1.5 L/ha were not tested).

Concerning soil mesofauna, earthworms (*Eisenia fetida*) show little sensitivity towards epoxiconazole as the 14 d LC50 for earthworms, determined in a filter paper contact test, the 48 h LC50 was >1000 µg/cm^2 (Wang et al., 2012). When the exposure was done in an artificial soil, Wang et al. (2012) reported a 7 d LC50 of epoxiconazole of 356.8 mg/kg, and a 14 d LC50 of 333.1 mg/kg, while the DAR (2006b) reports no mortality up to 1000 mg/kg in a 14 d study with artificial soil. Under the same conditions, the main degradation product, 1,2,4-triazole, also induced no mortality up to 1000 mg/kg. Overall the available data indicate low toxicity of epoxiconazole to soil mesofauna and terrestrial invertebrates.

Effects assessment with other azoles

Acute toxicity values of other triazole fungicides for aquatic invertebrates appear to be in a similar range to epoxiconazole. For example, EC50 values of 1.5 mg/L (48 h EC50) for ketoconazole and *D. magna* (Haeba et al., 2008), 6.1 mg/L (48 h EC50) for triadimefon in black fly, *Simulus vitattum*, larvae (Kenneke et al., 2009), or 6.56 mg/L (48 h EC50) for propiconazole and *Gammarus pulex* (Nyman et al., 2012).

Chronic toxicity values of other pesticides acting through inhibition of Cyp51 range from 0.28 mg/L in the case of prochloraz to 1.5 mg/L for triadimefon (Hassold & Backhaus, 2009). Teratogenic effects of triadimefon were studied in embryos of the ascidian, *Phallusia mammillata*, and the median teratogenic concentration that caused 50% malformed larvae was 8.1 mg/L, while the LC50 was 50.9 mg/L (Pennati et al., 2006).

While earthworms are believed to be generally rather insensitive to pesticides, epigeic Collembola (springtails) are considered a sensitive species (Frampton et al., 2006). The toxicity of formulated products on springtails was tested on field studies, but the study sites had been treated with epoxiconazole for many years so that a pre-selection of the Collembola populations cannot be excluded (DAR, 2006b). Frampton & Wratten (2000) investigated the potential effects of foliar sprays of propiconazole and triadimenol; they found no negative effect of the fungicides on springtail activity, and an only transient effect on springtail abundance.

The mode of action of triazole fungicides in aquatic and terrestrial invertebrates is not known. Nyman et al. (2012) provide evidence that propiconazole, under acute exposure conditions, shows baseline toxicity. Also alterations in energy metabolism (Sancho et al., 2009), glutathione-S-transferase (Johansen et al., 2007) or the appearance of toxic metabolites from triazole metabolism (Kenneke et al., 2009) have been suggested as processes contributing to triazole toxicity in invertebrates. Whether triazoles may have an endocrine-disrupting mode of action in invertebrates is discussed below.

Endocrine disrupting activities of triazole fungicides in invertebrates

The critical question is if P450-catalyzed reactions play a role in the endocrine systems of invertebrates, and if triazole fungicides such as epoxiconazole could inhibit those enzymes. Molecular and biochemical evidence of P450 enzymes have been demonstrated in all larger phyla of invertebrates, including Cnidaria, Annelida, Mollusca, Arthropoda and Echinodermata (Baldwin et al., 2009; Lee, 1998; Rewitz et al., 2006a; Snyder, 2000). Comparable to vertebrates, the main functions of invertebrate P450 enzymes are either catabolism of lipophilic endogenous and exogenous substrates, or the biosynthesis of signaling molecules including steroids (Rewitz et al., 2006b). Steroids with known hormonal activity in invertebrates include ecdysteroids and, possibly, vertebrate-type sex steroids. The synthesis of ecdysteroids by arthropods involves P450 enzymes. Ecdysteroids in concert with Juvenile Hormone (insects) or methylfarnesoate (crustaceans) orchestrate the periodic molting process, i.e. the replacement of the cuticle, as it takes place during growth, reproduction and metamorphosis of arthropods (Dubrovsky, 2005; LaFont, 2000). The active hormone, 20-hydroxy-ecdysone (20E), which binds to the ecdysteroid receptor, is generated from ecdysone (E), which in turn is synthesized *de novo* from dietary cholesterol or phytosterols (Gilbert, 2004; Rewitz et al., 2006a).

The capacity of invertebrates to synthesize vertebrate-type sex steroids is controversial. For molluscs, evidence has been presented that they are able to execute major enzymatic steps of steroidogenesis including conversion of cholesterol into pregnenolone (side chain cleavage) and aromatization (Janer & Porte, 2007; Lafont & Mathieu, 2007) and these enzymatic reactions are catalyzed by P450 enzymes (Le Curieux-Belfond et al., 2001; Matsumoto et al., 1997; Osada et al., 2004). What is lacking to date is genomic information on the presence of orthologs of steroid-synthesizing P450 enzymes in molluscs. Tiwary and Li (2009) reported identification of aromatase genes throughout the Eumetazoa, including invertebrates, but this conflicts with other authors who found no evidence for *Cyp*19 orthologous genes outside the chordate/vertebrate lineage (Markov et al., 2009; Reitzel & Tarrant, 2010). Thus the question of the presence of P450-catalyzed sex steroid synthesis in mollusks remains controversial as does the physiological functions of androgens and estrogens (Segner et al., 2003).

Given the evidence that P450 enzymes of invertebrates are involved (i) in the production of ecdysteroid hormones, which

regulate molting and associated processes in development, growth and reproduction, and possibly in (ii) the production of vertebrate-type sex steroids of uncertain physiological function in mollusks, the possibility exists that epoxiconazole and other Cyp-inhibiting azoles could contribute to endocrine disruption in invertebrates by interfering with hormone-synthesizing P450s. Generally, only few studies investigated if chemically induced disturbances of development or reproduction are related to an effect of these compounds on P450-mediated hormone metabolism.

Several azole compounds, in particular imidazoles, are known as potent inhibitors of ecdysteroid synthesis, and arthropods exposed to these compounds show disturbed development and reproduction (e.g. Amrani et al., 2004; Hassold & Backhaus, 2009). Fadrozole treatment at concentrations of 50 µg/L and higher of the mollusk *Potamophygus antipodarum*, resulted in induction of gonad histopathology, altered estrogen/androgen ratios and disturbance of reproduction (Gust et al., 2010). Triazole fungicides can also inhibit ecdysone-20-monooxygenase activity (Kenneke et al., 2009). There are a number of studies reporting developmental and reproductive changes in invertebrates exposed to triazole fungicides, e.g. the effect of epoxiconazole on emergence of *C. riparius* (see above), but these studies did not examine whether the developmental and reproductive effects were associated with an alteration in ecdysteroid or sex steroid metabolism (Figure 9).

Exposure and risk assessment for invertebrates

Concerning the aquatic environment, the exposure situation has been discussed above in the section on fish. In soil, epoxiconazole shows medium mobility and slow degradation. In alkaline to slightly acidic soils, epoxiconazole degradation appears to be enantioselective, whereas in more acidic soils, epoxiconazole enantiomers are degraded at similar rates (overall half-lives 78–184 d) (Buerge et al., 2006). A geometric mean half-life of 7 d at 20 °C corrected for temperature and soil moisture has been recommended to assess degradation behavior of epoxiconazole in the soil (DAR, 2006a). The main metabolite, which arises in only minor quantities from epoxiconazole metabolism in the soil (<10% of the parent compound), is 1,2,4-triazole (DAR, 2006a). Given the slow degradation of epoxiconazole in soil, the compound may accumulate over time. Field studies following the accumulation of epoxiconazole for 3–9 years at application rates of 312 to 437 g/ha revealed maximum residue levels in the top soil layers (0–25 cm) of 0.12 to 0.19 mg/kg, which indicates that an unacceptable accumulation in soil is unlikely (DAR, 2006a).

There appears to be no published information concerning triazole bioaccumulation in aquatic invertebrates, but for the triazole fungicide, propiconazole, low bioaccumulation factors were found in the aquatic invertebrate *Gammarus pulex* after multiple pulse exposures (Nyman et al., 2012). For *Daphnia*, long-term TER calculated under worst case assumptions and considering global maximum PEC values, meets trigger values indicating low risk. Similarly, for terrestrial arthropods such as bees or wasps, TER quotients also indicated low risk (DAR, 2006b).

Effects of epoxiconazole on algae and aquatic macrophytes

In evaluating toxicity data for algae and aquatic vascular plants, duckweed, *Lemna* sp, appears to be clearly more sensitive than aquatic invertebrates and algae (Figure 10). Even if the test with duckweed is performed in the presence of sediment (instead of water only, as in the standard test procedure) in order to mimic a more realistic exposure scenario, *Lemna* still shows very high sensitivity (DAR, 2006b). Duckweed is also more sensitive than algae (14 d EC50 biomass duckweed = 0.0081 mg/L, 72 h EC50 biomass algal *Ankistrodemus bibraianus* = 1.19 mg/L) (DAR, 2006b), and clearly more sensitive than any aquatic vertebrate species. Thus, in a conventional risk assessment approach, the toxicity values of duckweed would be of key importance both for classification and labeling and for the derivation of environmentally safe levels.

The high toxicity of epoxiconazole to *Lemna* appears to not be a singular event, but a general feature of triazoles. The Syngenta factsheet for cyproconazole (http://www.syngenta-cropprotection.com/Env_Stewardship/futuretopics/Cypro8-16-05.pdf, obtained June 1, 2013) reports an EC50 for duckweed of 0.070 ppm, which is in the same range as the algae EC50 of 0.026 ppm, but considerably lower than the EC_{50} values for invertebrates (2.6–26 ppm) or fish (19–21 ppm). Likewise, the factsheet of Nufarm for tebuconazole (http://www.nufarm.co.nz/Assets/18398/1/Hornet_430SC_SDS.pdf, obtained June 1, 2013) shows an EC50 for *Lemna gibba* of 0.1444 mg/L, while the algal EC50 is 3.8 mg/L, the *Daphnia* 48 h LC50 is 4 mg/L, and the fish 96 h LC50 is 4.4 mg/L. The Massachusetts Department of Agriculture (2012) reports LC50 values of paclobutrazol to *Daphnia magna*, mysid shrimps, and oyster larvae in the range of <9–35 mg/L, a 22 d chronic NOEC for *Daphnia magna* of 0.32 mg/L, a 96 h EC_{50} value in algae between 7.2 and >15.2 mg/L, whereas the 7 d EC50 for duckweed were between 0.0082 and 0.0283 mg/L. Penconazole is reported to have acute LC50 values for fish in the range of 1.13 to

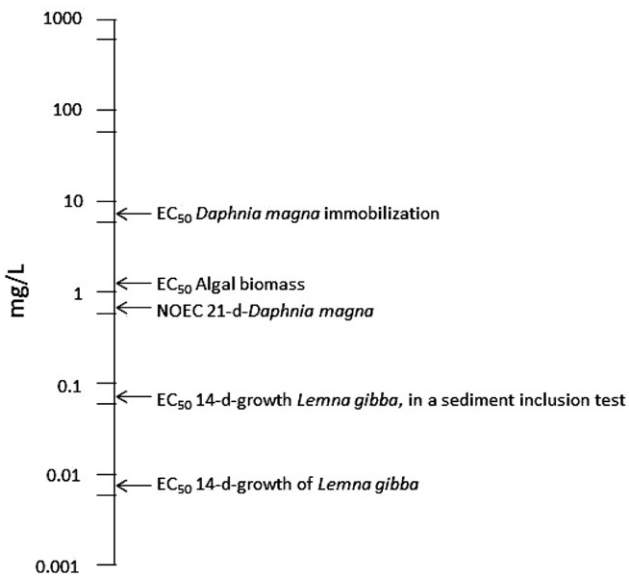

Figure 10. Effect concentration range of triazoles in (aquatic) invertebrates, algae and aquatic macrophytes.

3.8 mg/L, a chronic fish NOEC of 0.32 mg/L, *Daphnia magna* EC_{50} of 6.75 mg/L, a *Daphnia* 21 d NOEC of 0.069 mg/L, a 72 h alga EC50 of 4.9 mg/L. and a 14 d *Lemna* EC_{50} of 0.22 mg/L and a NOEC of 0.87 mg/L (EFSA, 2012). Taken together, these data point to duckweed as one of the more sensitive species to triazole activity. Algae, the second primary producer in aquatic ecosystems, appear to be less sensitive than *Lemna*. This applies as well for dinoflagellate microalgae which were used to assess genotoxic activity of epoxiconazole: the LOEC for genotoxic effects in dinoflagellates was 0.1 mg/L, and the NOEC was 0.01 mg/L (Akcha et al., 2008).

The question is whether there exists evidence that duckweed is generally a very sensitive genus to pesticides or to triazoles in particular. Duckweeds (mostly *L. minor* and *L.gibba*) are the only macrophytes used in the lower tiers of environmental risk assessment in North America, Europe and elsewhere. Duckweed testing has some peculiarities in that (i) duckweed does not root in sediment, (ii) duckweed is a monocot, so it may not be representative for dicot plants (Crespo et al., 2002) and (iii) duckweed tests are performed under eutrophic conditions, which may not be representative of oligo- or mesotrophic conditions (Rentz & Hanson, 2009) and therefore, the sensitivity of *Lemna* tests has been questioned (Fairchild et al., 1998; Rentz & Hanson, 2009; Van den Brink et al., 2006). Systematic comparisons of the sensitivity of duckweed versus other vascular plants, however, do not indicate a generally lower or higher sensitivity of duckweed. For instance, comparing the sensitivity of duckweed and algae (*Selenastrum capricormutum*) to 16 herbicides did not find a consistent difference between the algae and the vascular plants*: Lemna* was more sensitive to sulfonylureas and pyridines, while *Selenastrum* was more sensitive to thiocarbamates and triazines (Fairchild et al., 1997).Cedergreen & Streibig (2005) reported that *Lemna* and the algal species *Psuedokirchnerella subcapitata* showed comparable sensitivity to a series of herbicides, except compounds with a pKa < 5, for which *Lemna* showed an up to 1000-fold higher sensitivity. When Giddings et al. (2013) analyzed species sensitivity distributions for 11 herbicides and three fungicides, they found *Lemna* to be among the most sensitive macrophyte species for approximately half of the compounds. Overall, the available data suggest that the high sensitivity of *Lemna* to triazoles is not related to a generally high sensitivity of this species to all kinds of pesticides, but that this may indeed be a specific feature for triazole compounds.

Triazoles are potent inhibitors of elongation growth in vascular plants (Davis et al., 1991; Luster & Miller, 1993), and they are used as such in agricultural and horticultural practices. The suppression of growth by triazoles occurs because they block three P450-catalyzed steps in the terpenoid pathway for the production of the plant hormone gibberellins, which stimulates cell elongation. As the *Lemna* test measures foliage growth, its high sensitivity to triazoles may be caused by an inhibitory effect of triazoles on gibberellin synthesis.

The lower sensitivity of algae compared to *Lemna* might be explained by the fact that they do not rely on the gibberellin system, and the triazoles exhibit toxicity through alternative modes of action. For instance, for nitrogen-fixing cyanobacteria, toxicity of triazoles has been related to inhibition of nitrogen fixation (Kumar, 2010). Toxic effects in algae were also related to disturbances in amino acid metabolism and chlorophyll synthesis (Siegel & Gentile, 1966; Wolf, 1962).

Risk assessment for algae and aquatic plants

Aquatic exposure to epoxiconazole and other azoles has been discussed above. Comparing measured or predicted environmental concentrations of epoxiconazole and effect concentrations, as shown in Figure 11, indicates that they are not overlapping but still fairly close to each other. TER calculations for *Lemna* (DAR, 2006b) as the most sensitive species, results in values, depending on the specific exposure scenario, between 6.7 and 245, part of the range of which would be below the trigger value of 10, which is considered to represent a potential hazard to the exposed biota. Thus, a more refined risk assessment should be considered.

Alternate ecotoxicological risk assessment approaches

Instead of TER values based on a single species test, Species Sensitivity Distributions (SSD) can also be used to assess the ecological risk of chemicals. Maltby et al. (2009) constructed acute toxicity data SSDs for pesticides including fungicides. From the single species SSD, the authors derived a hazardous concentration for 5% of the species (HC_5) and compared it to an ''ecosystem threshold'' value ($LOEC/NOEC_{eco}$) which was calculated from multispecies data collated from 21 studies with 12 fungicides. The results from this comparison showed that the derived HC5 values were always lower than the $LOEC_{eco}$, and lower than the $NOEC_{eco}$ for 3 of the 9 fungicides for which a $NOEC_{eco}$ could be calculated.

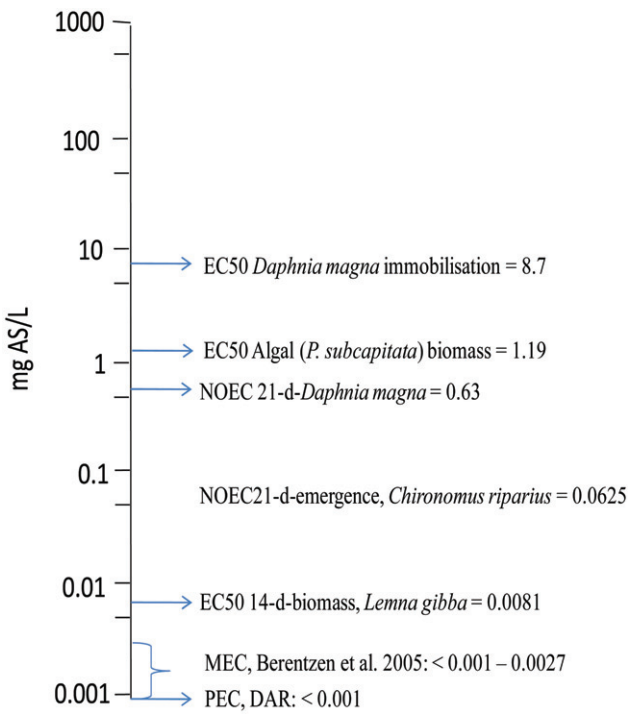

Figure 11. Predicted (PEC) and measured environmental concentration (MEC) of epoxiconazole versus effects levels in invertebrates and plants.

For epoxiconazole, the authors constructed an SSD, with an HC_5 of 14 µg/L. It was, however, not possible to calculate an ecological threshold value for epoxiconazole so that it remains open if the HC_5 of 14 µg/L would be protective of adverse ecological effects.

The limited availability of toxicity data is a major problem in the risk assessment of epoxiconazole for invertebrates and primary producers. For such a situation, Brock et al. (2011) took geometric mean values of the available toxicity data and divided them by 100 (for acute toxicity data) or 10 (for chronic toxicity data) to obtain a RAC (regulatory acceptable concentration) value which could then be compared to Predicted Environmental Concentrations (PEC) and Maximum Acceptable Concentration (MAC) values. For epoxiconazole, Maltby et al. (2009) calculated an EC50 of 5620 µg/L for fish, 740 µg/L for invertebrates, and 600 µg/L for primary producers, which would result in RAC values of 56.2, 7.4 and 6 µg/L for fish, invertebrates and primary producers, respectively. These values would be above PEC and MAC levels of epoxiconazole.

The various approaches addressed above point to low risk of epoxiconazole to aquatic invertebrates and primary producers. The same applies for terrestrial invertebrates. The conclusions are unlikely to change if more information on endocrine-disrupting effects of epoxiconazole in invertebrates were available. Epoxiconazole-induced disruption of ecdysteroid or sex steroid metabolism would lead to altered development and reproduction of invertebrates. Effect concentrations for these endpoints exist and were already included in the TER calculations, which led to the conclusion that the risk of epoxiconazole for invertebrates is low. The possibility remains that fungicides could have indirect effects on invertebrates through effects on aquatic microbial communities and leaf decomposition as shown by Rasmussen et al. (2012). However the concentrations used in that study (50 and 500 µg/L) were clearly higher than realistic environmental concentrations of epoxiconazole. Another question is whether decomposition, a process in which aquatic fungi play an important role, might be particularly at risk from epoxiconazole. Interestingly, Maltby et al. (2009) in four multispecies studies with fungicides (carbendazim, fluazinam, triphenyltin acetate, tolyfluanid) found no case in which decomposition was the most sensitive endpoint.

Discussion

This review explored the issue of hazard based approaches using endocrine disruption as an endpoint versus conventional risk assessment. In this review the use of azole fungicides/antifungals in general, and in particular epoxiconazole used under standard agricultural applications as a case study, was determined by a number of factors. These factors included the diversity of this class of compounds, the presence of mechanisms of toxicity that can logically include endocrine disruption, their wide range of uses, a range of human exposure levels spanning intentional (from therapeutic antifungals) to unintentional (from agricultural fungicides), and the inclusion of compounds of high agricultural importance. This breadth also means that there is a considerable body of information on which to base the discussions and conclusions that have been reached. While we will acknowledge that there are data gaps/deficiencies, there are none that prevent a meaningful, evidence-based analysis. It needs to be recognized that epoxiconazole may not be completely representative of the azole class of fungicides because of the differences among the azoles in disposition and metabolism, as well as potency for primary and secondary target molecules, such as the CYPs. It also needs to be recognized that in some respects azoles may not be completely representative of other environmental endocrine disruptors because of the diversity of molecular targets that could be affected by different chemicals.

As this review demonstrates, epoxiconazole and other azole fungicides have potential endocrine disrupting properties as a direct consequence of their ability to interact with P450 enzymes that are involved in reproductive hormone metabolism, most notably CYP19 (aromatase). The scale and affinity of this interaction clearly varies according to the P450 enzyme in question and according to the azole compound; for example, their ability to inhibit CYP19 in vitro ranges across 4 orders of magnitude. This leads to one aspect of uncertainty in relating the toxicity of epoxiconazole with other azoles, i.e. whether other azoles can be used as surrogates for understanding the actions of epoxiconazole. However for the purposes of this review we have done so as, in general, the azoles as a class exhibit similar effects on critical P450s such as aromatase.

As indicated earlier the dosage levels in rats causing endocrine-mediated toxicities (i.e. post-implantation losses, fetal resorptions, increased anogenital distance in female pups) are 50–180 mg/kg for about 1.5–2 weeks during pregnancy. In contrast liver toxicity in animal studies occurred at 7 mg/kg/d, so at least a 7-fold lower dosage. Similarly, in studies with birds and fish, effects on behavior and/or growth were generally more sensitive than reproductive responses. Thus the endpoints used in conventional risk assessments would be more protective of the health of humans and wildlife.

Using a solely hazard-based approach, epoxiconazole as an example, would have to be classified as an endocrine disruptor (Ankley et al., 2003). If this were used as the basis for regulation, three key pieces of information that are already available would be ignored. First, the evidence that human exposure to epoxiconazole, even in a worst-case scenario involving applicators, falls at least 100 000-fold below the threshold for adverse effects that have been attributed to endocrine disruption (e.g. applicator level of 0.001 mg/kg compared to fetal loss in pregnancy occurring at 100 mg/kg; Figure 5). For the general population, this margin of safety is expected to be even 10- to 100-fold greater. While the margins of safety are less for wildlife including invertebrates and most plants, conventional risk assessment based on the most sensitive endpoints also points to negligible risk associated with epoxiconazole exposure. Second, a hazard-based approach would not take into account that one of the adverse effects attributed to endocrine disruption from the laboratory rodent studies, i.e. fetal loss in pregnancy, is probably not human-relevant as the mechanism involved in pregnancy disruption is specific to rodent reproductive physiology (i.e. the fetal loss in rodents results from Cyp19

inhibition in the corpora lutea of the dam preventing necessary maternal estrogen from maintaining the pregnancy, whereas the fetus/placenta produces the estrogen in the human, and estrogen is less important in the human for pregnancy maintenance). In studies with birds and fish, effects on behavior and/or growth were generally more sensitive than endocrine response which reinforces the concept that emphasis should be placed on assessing the most sensitive responses. Third, this approach would ignore the extremely large database of information that derives from study of humans exposed intentionally during pregnancy to therapeutic azole antifungal compounds, which identify that even high exposures to members of the azole class are without adverse effects, except in extreme (life-threatening) circumstances; the fact that the latter adverse effects occur at dosages at least 1 250-fold higher than the ADI for human exposure to epoxiconazole (comparing the 10 mg/kg concentration of fluconazole that induced human facial abnormalities in offspring to the ADI of 0.008 mg/kg; Figure 5). It is important to note that the ADI already incorporates a 250-fold safety factor which provides reassurance of a large, evidence-based margin of safety, even considering potential differences in potency among the various azoles (Figure 5). Similarly, considerable margins of safety are evident from studies with wildlife including invertebrates and most plants. Viewed together, these facts suggest that in the case of epoxiconazole (but probably applicable in general to other azole fungicides), use of a hazard-based approach, particularly emphasizing endocrine disruption endpoints, to regulation would effectively ignore most of the available, relevant evidence and would thus not be scientifically grounded (Rhomberg et al., 2012).This conclusion draws some parallels with other suspected EDCs. For example in the assessment of bisphenol A, the critical effects that were selected as the basis for the TDI were changes in body and organ weights in adult and offspring rats and liver effects in adult mice, not endocrine-related effects (EFSA, 2006).

A review of the data on this case study with epoxiconazole reveals through both human and ecological risk assessment that agricultural use under standard applications can be done effectively and safely. Epoxiconazole exposure is unusually low because of its high efficacy in preventing fungal disease in the crops on which it is used; as noted earlier, normal practice involves spraying of only 125 g/hectare on two occasions during the growing season. Therefore, the large margin of safety (human) as well as the comparatively low sensitivity of endocrine endpoints (wildlife) is evident for epoxiconazole (Figures 6–11), and may have application when studying many other endocrine disruptors. There may be some exceptions such as is the case for ethinylestradiol, which is also an environmental endocrine disruptor and where the primary action is based on the interaction with estrogen receptors. In such a case it may be appropriate to base the hazard assessment on endocrine function. Nevertheless, the present example demonstrates quite convincingly that endocrine disruption *per se* does not provide any simple, sensitive and more meaningful information on which to base evidence-based safety assessment than already exists in conventional risk assessment (Kendall et al., 2010), at least for epoxiconazole and other azole fungicides.

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Declaration of interest

The employment affiliation of the authors for their usual activities is as shown on the cover page. However, all of the authors participated as independent consultants in the review presented in this paper. Ronald J. Kendall is the owner of Ronald J. Kendall and Associates, Inc. (RJKA), an independent consulting firm serving both public and private clients. Ronald J. Kendall, as the principal in RJKA, from time to time engages scientific colleagues to assist in his advisory activities. The other five authors (Chambers, Greim, Segner, Sharpe and Van Der Kraak) were engaged by RJKA to assist in this review. RJKA was awarded a contract from BASF Corporation to establish an international SAP to evaluate potential endocrine effects of epoxiconazole and other azole fungicides of human or wildlife relevance under practical agricultural conditions.

BASF Corporation is an international developer, manufacturer and marketer of agricultural chemicals, including epoxiconazole. Janice E. Chambers declares she has also consulted for Dow Chemical Company. Ronald J. Kendall declares an ongoing consultancy with DuPont. Glen Van Der Kraak declares ongoing consultancy with Syngenta Crop Protection. Dow Chemical Company, DuPont and Syngenta Crop Protection Company are all developers, manufacturers and marketers of agricultural chemicals. None of the authors have appeared before any regulatory bodies concerning the compounds reviewed in this paper nor have they appeared as experts in litigation concerning these compounds. The authors functioned as a SAP in conducting their review. They independently determined the literature review strategy, reviewed the literature, conducted the data analysis and synthesis and drew the conclusions presented. The writing of the paper and its contents were exclusively their responsibility. The BASF Corporation was given the opportunity to review and offer comments on drafts of this report. The review comments were considered by the authors; however, the conclusions drawn are exclusively those of the authors. The authors are aware that BASF Corporation could use the report in support of regulatory actions around the world.

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