Prochloraz, CAS no. 67747-09-5

Synonyms: N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide.

Prochloraz (Figure 1) is a broad-spectrum imidazole fungicide. Prochloraz is widely used in gardening and agriculture. It is used on wheat, barley, mushrooms, cherries, turf on golf courses, and in flower production, for instance, in Ecuador, where roses are treated with prochloraz prior to export to the USA. Its fungicidal activity is due to inhibition of 14 alpha- demethylase (CYP 51), an enzyme required for the synthesis of fungal cell walls. Prochloraz is an agricultural imidiazol fungicide that inhibits a CYP enzyme involved in ergosterol synthesis, but has also been reported to inhibit other CYP enzymes, and to act as a potent aromatase inhibitor. This EU approval expires 31st December 2021. At the EU Member State level it has been approved in 25 EU countries (except Malta and Denmark) (EU, 2016).

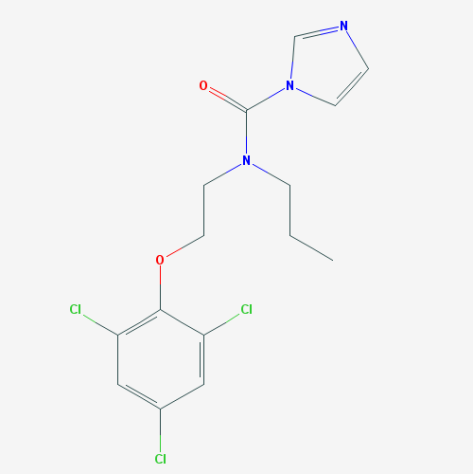

Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 In vitro information indicative of endocrine activity

Kjærstad et al. (2010)

Summary: In this study widely used conazole antifungals were tested for endocrine disruptive effects using a panel of *in vitro* assays. They all showed endocrine disrupting potential and ability to act via several different mechanisms. Overall the imidazoles conazole, ketoconazole, miconazole, prochloraz) were more potent than the triazoles (epoxiconazole, propiconazole, tebuconazole). The critical mechanism seems to be disturbance of steroid biosynthesis. In the H295R cell assay, the conazoles including prochloraz decreased dose-dependently the formation of estradiol and testosterone, and increased the concentration of progesterone. Effects at 0.01µM (lowest concentration tested). Maximum effects (at 3.0 µM) were less than 10% of the solvent control. This was indicating inhibition of enzymes involved in the conversion of progesterone to testosterone. Prochloraz was most potent. In the MCF- 7 cell proliferation assay, the conazoles showed anti-estrogenic effect, including aromatase inhibition, since they inhibited the response induced by both 17β-estradiol (incl. prochloraz) and testosterone (incl. prochloraz).

Study quality and assessment: The study is well-described. In this study prochloraz was assessed in a battery of *in vitro* tests. Purity (99.5%) and CAS no. is given and cytotoxicity was evaluated by using the Cytotox 96 Non-Radioactive Cytotoxicity assay. Overall the quality of the study is assessed to be high, and the study provides strong evidence for an anti-androgenic, anti-estrogenic effect and also aromatase inhibiting MoA of prochloraz.

Hecker et al. 2006
Summary: In this study an *in vitro* screening assay based on measuring alterations in hormone production was developed using the H295R human adrenocortical carcinoma cell line. The objective of the H295R Steroidogenesis Assay (later OECD TG 456) is to detect substances that affect production of estradiol (E2) and testosterone (T) and P(progesterone/pregnenolone). Prochloraz is included in this Test Guideline as a positive control as it is a strong inhibitor of steroid hormone secretion. The aim of the current study was to develop and standardize an *in vitro* Tier 1 screening assay using the H295R cell line to prioritize chemicals that act to alter hormone production. CAS and purity was not given in this study. Model chemicals with different modes of action on steroidogenic systems were tested in this study. Exposure to forskolin resulted in dose-dependent increases in all three hormones with the greatest relative increase being observed for E2. This differed from cells exposed to prochloraz (or ketoconazole) where P concentrations increased while T and E2 concentrations decreased in a dose-dependent manner at prochloraz concentrations greater than 0.03 and 0.003 μM, respectively. Based on these results, the H295R *in vitro* system has potential for high throughput screening to not only characterizes the effects of chemicals on endocrine systems but also to prioritize chemicals for additional testing.

Study quality and assessment: The study is well-described and prochloraz was included as positive control in this study (as later in the final OECD TG 456). More information on CAS-number would have been preferred and cytotoxic chemical concentrations were not included in the hormone concentration measurements. Overall the quality of the study is still assessed to be high, and the study provides strong evidence for an anti-estrogenic and anti-androgenic MoA of prochloraz.

Overall, a large battery of published *in vitro* screening studies indicates that prochloraz is able to induce endocrine imbalance by affecting the androgen and oestrogen receptor, to inhibit the aromatase activity and to interfere with steroidogenesis probably at the level of P450c17. A review of the endocrine-related data available for prochloraz has been conducted in GD 181 by OECD which led to the conclusion that “The combined dataset provides sufficient evidence of endocrine activity” (OECD 2012). Also the review by Melching-Kollmuss et al. (2017) gives an overview of *in vitro* assays on prochloraz.

4.10.3.3 In vivo effects with regard to an endocrine mode of action

Melching-Kollmuss et al. (2017)
Summary: This paper provides a good review of regulatory and peer reviewed studies of prochloraz. Prochloraz was administered per gavage in oil from gestation day (GD) 6 to postnatal day (PND) 83 to pregnant and lactating Wistar rats and their respective offspring, at doses of 0.01 mg/kg bw/day (acceptable daily intake of prochloraz), 5 mg/kg bw/day (expected no-observed-effect-level (NOEL)) and 30 mg/kg bw/day. At 30 mg/kg bw/day maternal and offspring effects (decreased viability, lower number of live offspring) were seen including a delayed entry into male puberty (plus one day) accompanied by lower male offspring body weights, increased ano-genital distance/index in females and (transiently) retained nipples in males at PND 12 (not seen at PND 20). The only finding at the expected NOEL was increased incidences of transiently retained nipples in males which the authors do not consider an adverse finding. No effects were seen in the low-dose group.

Study quality and assessment: The study is well-described and evaluates prochloraz at very low levels (ADI, NOEL, 6 times NOEL). The study is of moderate quality as it has some limitations. It is reported that 20 mated females per dose group are used. Unfortunately, it is unclear how many litters are evaluated for each endpoint. Additionally, it is not clear whether they make statistics (e.g. ano-genital distance (AGD)) on individual pups and take litter effect into account (as described) or if they are just looking at individual pups which is not nearly as sensitive. They find retained nipples at day 12-13. The authors do not believe that a significant incidence of preserved nipples is an adverse effect. However, it is possible to set a NOAEL on this finding according to OECD guidance documents. Overall the study provides strong evidence for adverse effects such as increased nipple retention (even at low doses and thereby an anti-androgenic MoA of prochloraz.

Blystone et al. (2007)

Summary: Prochloraz was administered by gavage to pregnant rats at doses of 0, 7.8, 15.6, 31.3, 62.5, and 125 mg/kg/day from gestational day 14 to 18. On gestational day 18, hormone production from ex vivo fetal testes was examined and prochloraz levels in amniotic fluid and maternal serum were measured. Fetal P and hydroxy-P production levels were increased significantly at every prochloraz dose, whereas T levels were significantly decreased only at the two high doses. These results suggested that prochloraz inhibits the conversion of P to T through the inhibition of CYP17. Prochloraz had no effect on testicular CYP17 gene expression (mRNA levels) but CYP17 hydroxylase activity was significantly inhibited when tested in vitro (Ki = 865 nM). Amniotic fluid prochloraz concentrations ranged from 78 to 1512 ppb and T production was reduced when prochloraz reached approximately 500 ppb, which compared favourably with the determined CYP17 hydroxylase Ki (326 ppb).

Study quality and assessment: The study is well-described and results demonstrate that prochloraz lowers testicular T synthesis by inhibiting CYP17 activity which likely contributes to the induced malformations in androgen-dependent tissues of male offspring. Overall the quality of the study is assessed to be high, and the study provides strong evidence for decreased testicular T synthesis and thereby an anti-androgenic MoA of prochloraz.

Laier et al. (2006)

Summary: Prochloraz was administered orally to pregnant rats at doses of 50 and 150 mg/kg/day, from gestational day 7 to postnatal day (PND) 16. Male and female offspring were examined, a subset of foetuses were examined after Caesarean section of dams at gestational day 21.

Prochloraz caused mild dysgenesis of the male external genitalia, reduced ano-genital distance (AGD) and retention of nipples in male pups. In female pups AGD was increased AGD. In male foetuses, testicular and plasma levels of testosterone (T) were decreased and levels of progesterone (P) increased. Immunohistochemistry of fetal testes showed increased expression of 17alphahydroxylase/ 17,20-lyase (P450c17) and a reduction in 17beta-hydroxysteroid dehydrogenase (type 10) expression. Increased expression of P450c17 mRNA was observed in fetal male adrenals, and the androgen-regulated genes ornithine decarboxylase, prostatic binding protein C3 as well as insulin-like growth factor I mRNA were reduced in ventral prostates at PND 16. These results indicate that reduced activity of P450c17 may be a primary cause of the disrupted fetal steroidogenesis and that altered androgen metabolism may also play a role

Study quality and assessment: The study is well-described and evaluates prochloraz at high doses. The study is of moderate quality as it uses small group sizes 5-8 litters per group. This study find decreased AGD (6-12%) in the males (from 50 and 150 mg/kg group respectively). This decrease in male AGD has not been seen in several other studies (Christiansen et al. 2009, Noriega et al 2005; Vinggaard et al. 2005; Melching-Kollmuss et al. 2017). The effects on NR and/or increase in female AGD have also been seen in several studies (Christiansen et al. 2009; Vinggaard et al 2005; Melching-Kollmuss et al 2017). Overall the study provides strong evidence for adverse effects such as dysgenesis and increased nipple retention and thereby an anti-androgenic MoA of prochloraz.

Noriega et al. (2005)

Summary: Prochloraz was administered by gavage to pregnant rats at doses of 31.25, 62.5, 125, and 250 mg/kg/day from gestational day 14 to 18. Prochloraz delayed delivery in a dose-dependent manner and resulted in pup mortalities at the two highest doses. In male offspring, anogenital distance (AGD) adjusted\for body weight was not affected, but in females adjusted AGD was increased at 250 mg/kg. In females VO (vaginal opening) was unaffected. Nipple retention (NR) was observed in males at 13 days of age at frequencies of 31%, 43%, 41%, and 71% in the lowest-dose to highest-dose groups, respectively. Weights of sexual accessory tissues showed dose dependent reductions. Hypospadias and vaginal pouches were noted in all males treated with 250 mg/kg, whereas those defects were observed in 12.5% and 6.25%, respectively, of males treated with 125 mg/kg. Treatment did not affect age of PPS in animals without penile malformations. Despite severe malformations in males, no malformations were noted in females.

Study quality and assessment: The study is well-described and evaluates prochloraz at high doses. The study is of moderate quality as it uses very high doses (general toxic doses). This study find decreased absolute AGD at 125 and 250 mg/kg, but no effect on AGD when body weight was included as covariate (which is normally done). The effects on increased NR seen at 125 and 250 mg/kg, but not at the lower doses 31 and 63 mg/kg have also been seen in several other studies (Christiansen et al 2009; Vinggaard et al 2005; Melching-Kollmuss et al 2017). Overall the study provides strong evidence for adverse effects such as increased nipple retention and thereby an anti-androgenic MoA of prochloraz.

Vinggaard et al. (2005)

Summary: Prochloraz was administered orally to pregnant rats at a dose of 30 mg/kg/day, from gestational day 7 to postnatal day (PND) 16. Male and female offspring were examined, a subset of foetuses were examined after Caesarean section of dams at gestational day 21. Gestational length was increased by prochloraz. Plasma and testicular T levels in gestational day 21 male foetuses were reduced. Chemical analysis of the rat breast milk showed that prochloraz was transferred to the milk. In males nipple retention was increased, and the bulbourethral gland weight was decreased, whereas other reproductive organs were unaffected. CYP1A activities in livers were induced by prochloraz. Behavioural studies showed that the activity level and sweet preference of adult males were significantly increased.

Study quality and assessment: The study is well-described and evaluates prochloraz at lower doses and in many litters per group (N=16). The study is of high quality and find increased number if retained nipples and also behavioural effects. Overall these results provide strong evidence for

adverse effects and that prochloraz feminizes the male offspring after perinatal exposure. Moreover this study indicates that these effects are due, at least in part, to diminished fetal steroidogenesis.

Prochloraz has been tested in a full set of regulatory toxicological studies including two multi-generation reproductive toxicity studies, which was performed according or comparable to the US EPA OPPTS 870-3380 or OECD TG 416 (Two generation test) These TG 416 studies was made before the update in 2001, so none of them were to current standards (OECD, 2012b)

These regulatory toxicological studies are summarized below and in the DAR of Prochloraz and in EFSA conclusion (EFSA, 2007; EFSA, 2011).

Prochloraz was evaluated in two key two-generation toxicity studies from 1993 and 1982 where overall reproductive performance was impaired following prochloraz administration to rats (Cozens et al. (1982) as summarized in DAR and Reader et al. (1993) as summarized in EFSA conclusion). Effects on reduction in body weight and body weight gain, increased liver weight and deaths were associated with dystocia and extended gestation length. Developmental toxicity was observed as reduced mean litter size, increased total litter loss, reduced live birth index, impaired growth and adverse effects on organ weights. In the 1993 study the agreed parental and reproductive NOAEL was 50 ppm (2.26 mg/kg bw/d), and the offspring NOAEL is 150 ppm (6.58 mg/kg bw/d). In the study from 1983 the agreed parental NOAEL is 150 ppm (13 mg/kg bw/d), the reproductive NOAEL is 37.5 ppm (3.1 mg/kg bw/d), and the offspring NOAEL is 150 ppm (13 mg/kg bw/d) (EFSA conclusion 2011).

In the developmental toxicity studies, there was no evidence of teratogenicity, and the relevant maternal and developmental NOAELs are 25 mg/kg bw/d for the rat and 40 mg/kg bw/d for the rabbit.

Prochloraz has been tested in these regulatory toxicological studies as summarised above. These guidelines, however, precede OECD harmonization lack specific parameters to sensitive to endocrine disruption and to identify anti-androgenicity (e.g., sperm parameter, onset of puberty, AGD and nipple retention). The effect on dystocia was the only endocrine endpoint found in these studies.

As reported in published papers (some of them mentioned above), rats displayed typical signs of anti-androgenicity after treatment with prochloraz, like retained nipples, reduced testosterone, increased progesterone levels, reduced male reproductive organ weights and delayed entries into male puberty (Blystone et al. 2007; Laier et al. 2006; Vinggaard et al. 2002). A NOAEL identified for the most sensitive endpoint retained nipples was 5 mg/kg bw/day in rats was determined by Christiansen et al. (2009). A summary of the findings seen in both regulatory studies and in *in vivo* studies published in peer-reviewed journals is provided in GD 181 (OECD 2012b) and the review by Melching-Kollmuss et al. (2017).

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Prochloraz is recognized as an endocrine disrupter as there is strong evidence from *in vitro* assays that the substance acts by multiple mechanisms of action in non-target species including inhibition of enzymes of steroidogenesis (CYP 19, CYP 17 and 5 α-reductase) and AR antagonism. Moreover, *in vivo* studies have shown adverse effect on male reproductive development as development of several androgen-dependent tissues has been affected as summarized below.

Prochloraz has been shown to react through several endocrine disrupting mechanisms (Vinggaard et al., 2005; Vinggaard et al. 2006). Moreover *in vivo* prochloraz can affect the development of several androgen-dependent tissues (Vinggaard et al., 2002; Vinggaard et al. 2005, Laier et al. 2006, Taxvig et al. 2008). Common features for the azole fungicides are that they increase gestational length and affect steroid hormone levels in fetuses and/or dams. In the majority of studies, male offspring, exposed *in utero* to prochloraz often showed no statistically significant changes in anogenital distance (AGD) with doses from 25-150 mg/kg but find significant nipple retention (NR) (Vinggaard et al. 2005; Christiansen et al. 2009; Noriega et al. 2005 and Melching-Kollmuss et al. 2017). One study has found both a decrease in male AGD at 50 and 150 mg/kg and also increased NR (Laier et al. 2006). Several studies find an increase in female AGD after *in utero* exposure to prochloraz (Laier et al. 2006; Melching-Kollmuss et al. 2017).

In addition to its anti- androgenic MoA, interference with testosterone synthesis and steroidogenic MoA, prochloraz has also been shown to affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain (Vinggaard et al. 2005).

The results of several *in vitro* assays, which have been published over the last 15 years, point out that prochloraz has endocrine mode(s) of action. These *in vitro* screening studies were carried out in various test systems such as steroid hormone synthesis assay (e.g., OECD TG 456 (2011)). And in the final TG 456 prochloraz is used as a positive control as it is a strong inhibitor of steroid hormone secretion (OECD, 2011). Prochloraz was found to be able to interact with estrogen and/or androgen receptors, with aromatase and with the steroid hormones (Andersen et al. 2002; Birkhøj et al. 2004; Grünfeld and Bonefeld-Jorgensen 2004; Hecker et al. 2006; Kojima et al. 2004; Laier et al. 2006; Sanderson et al. 2002; Trösken et al. 2004; Vinggaard et al. 2000, 2002, 2005). More recent studies suggest that prochloraz interferes with steroidogenesis in *in vitro* systems by inhibition of P450c17 (17α-hydroxylase and 17,20-lyase) (Nielsen et al. 2012). Cortisol and corticosterone levels were shown to decrease after exposure of H295R cells to prochloraz (Winther et al. 2013; Ohlsson et al. 2010). In some of these studies also gene expression of relevant steroidogenesis genes were investigated: H295R cells exposed to 0.03 μM prochloraz showed decreased expressions of some of the genes involved in steroidogenesis were seen (Ohlsson et al. (2009); Ohlsson et al. 2010).

Overall, this battery of *in vitro* screening studies shows that prochloraz is able to induce hormonal imbalance by affecting both the androgen and estrogen receptor, to inhibit the aromatase activity and to interfere with steroidogenesis probably at the level of P450c17.

In OECD GD 181 (OECD, 2012b) prochloraz was one of the cases used for the OECD GD 150 (OECD, 2012a). In GD 181 a review of the endocrine-related data available for prochloraz was collected (on both in vitro, mammals and fish) and this to evaluate whether the conclusions and next

steps recommended in the guidance document for identification of ED was sensible and helpful when assessed in light of comprehensive datasets

In GD 181 it says: "*The combined dataset indicates that the ER and AR antagonism and S disruption shown in vitro also occur in vivo in mammals and fish. The antagonist response in the Hershberger assay provides confirmation that this mechanism may act in vivo, whilst the positive results in male PP assay (Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats) and fish assays suggest that A, S or E modalities may be responsible for the effects seen on endocrine endpoints. The combined dataset provides sufficient evidence of endocrine activity*". Moreover in relation to the generation studies: "*Although the 2-generation study was negative for endocrine effects, the combined dataset provides sufficient evidence of concern for endocrine activity in mammals. NOAELs for reproduction and development could be derived from the combined dataset, thus avoiding further testing. The combined dataset indicates that the ER and AR antagonism and S disruption shown in vitro also occur in vivo in mammalian species. These effects also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different*."

The total evidence for adverse effects of prochloraz is strong (Table 2), the evidence for an anti-androgenic MoA (incl. anti-estrogenic MoA) of prochloraz and inhibition of enzymes in steroidogenesis is strong (Table 1) and the evidence for a plausible link between the MoA and adverse effects is also strong.

In conclusion, prochloraz meet the WHO definition of an endocrine disruptor.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of prochloraz

Reference	**MoA**		**Quality of study**	**Evidence for ED MoA**
	In vitro	*In vivo*		
OECD, 2012b, Review	The combined dataset provides sufficient evidence of endocrine activity.	The combined dataset indicates that the ER (estrogen receptor) and AR (androgen receptor) antagonism and S (steroidogenic) disruption shown *in vitro* also occur *in vivo* in mammals and fish. The combined dataset provides sufficient evidence of endocrine activity.	High	Strong

Table 2. Overview of potential endocrine-related adverse effects of prochloraz

Reference	**Species, n**	**Adverse effects**	**Quality of study**	**Evidence for adverse effects**
OECD, 2012b Review	Rats	Malformed genitalia in males, altered AGD (females), caused nipple retention in males, decreased serum Testosterone, reduced sexual accessory tissue weight males, sweet preference increased males.	High	Strong

References

Andersen, H.R., Vinggaard, A.M., Rasmussen, T.H., Gjermandsen, I.M. and Bonefeld-Jorgensen, E.C. (2002) 'Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro.', *Toxicol Appl Pharmacol 179:1–12*

Birkhøj, M., Nellemann, C., Jarfelt, K., Jacobsen, H., Andersen, HR., Dalgaard, M. and Vinggaard, A.M. (2004) 'The combined anti-androgenic effects of five commonly used pesticides.', *Toxicol Appl Pharmacol. 201(1):10–20*

Blystone, C.R., Lambritth, C.S., Howdeshell, K.L., Furr, J., Sternberg, R.M., Butterworth, B.C., Durhan, E.J., Makynen, E.A., Ankley, G.T., Wilson, V.S., LeBlanc, G.A. and Gray, L.E. (2007) 'Sensitivity of fetal rat testicular steroidogenesis to maternal prochloraz exposure and the underlying mechanism of inhibition.', *Toxicol Sci 97(2):512–519*

Christiansen, S., Scholze, M., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Kortenkamp, A. and Hass, U. (2009) 'Synergistic disruption of external male sex organ development by a mixture of four anti-androgens.' *Environ Health Perspect 117:1839–1846*

EFSA, 2007 DAR, Draft Assessment Report Prochloraz: http://dar.efsa.europe.eu/darweb/provision

EFSA (2011), Review report for the active substance prochloraz, finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 27 September 2011, in view of the approval of prochloraz as active substances in accordance with Regulation (EC) No 1107/2009, p.1-8.

EU, 2016, EU Pesticides database, prochloraz: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.detail&language=EN&selectedID=1753

Grünfeld, H.T. and Bonefeld-Jorgensen, E.C. (2004) 'Effect of in vitro estrogenic pesticides on human oestrogen receptor α and β mRNA levels.', *Toxicol Lett 151:467–480*

Hecker, M.(1), Newsted, J.L., Murphy, M.B., Higley, E.B., Jones, P.D., Wu, R. and Giesy, J.(2006) 'Human adrenocarcinoma (H295R) cells for rapid *in vitro* determination of effects on steroidogenesis: hormone production.', *Toxicol Appl Pharmacol.217(1), pp.114-24.*

Kjærstad, M.B., Taxvig, C., Nellemann, C., Vinggaard, A.M., Andersen, H.R. (2010) 'Endocrine disrupting effects *in vitro* of conazole antifungals used as pesticides and pharmaceuticals.' *Repro.Toxicol.30(4), pp.573-82,* doi: 10.1016/j.reprotox.2010.07.009.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K. and Kobayashi, K. (2004) 'Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells.', *Environ.Health.Perspect.112, pp.524–531*

Laier, P., Metzdorff, S.B., Borch, J., Hagen, M.L., Hass, U., Christiansen, S., Axelstad, M., Kledal, T., Dalgaard, M., McKinnell, C., Brokken, L.J. and Vinggaard, A.M. (2006) 'Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz.', *Toxicology and Applied Pharmacology.213, pp.160–71.*

Melching-Kollmuss, S., Fussell, K.C., Schneider, S., Buesen, R., Groeters, S., Strauss, V. and Van Ravenzwaay, B. (2017) 'Comparing effect levels of regulatory studies with endpoints derived in targeted anti-androgenic studies: example prochloraz.', *Archives of Toxicology.91(1), pp.143-162.* doi: 10.1007/s00204-016-1678-y.

Nielsen, F.K., Hansen, C.H., Fey, J.A., Hansen, M., Jacobsen, N.W., Halling- Sorensen, B., Björklund, E. and Styrishave, B. (2012) 'H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones.', *Toxico. In Vitro 26, pp.343–350*

Noriega, N.C., Ostby, J., Lambright, C., Wilson, V.S., Gray, Jr.L.E. (2005) 'Late gestational exposure to the fungicide prochloraz delays the onset of parturition and causes reproductive malformations in male but not female rat offspring.', *Biology of Reproduction.72, pp.1324–1335.*

OECD (2011), H295R Steroidogenesis Assay, OECD Environmental Health and Safety Publications, Series on Testing and Assessment no. 456., Organisation for Economic Cooperation and Development, Paris.

OECD (2012a), Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. OECD Environmental Health and Safety Publications, Series on Testing and Assessment no. 150., Organisation for Economic Cooperation and Development, Paris.

OECD (2012b), Guidance document (GD) on standardised test guidelines for evaluating chemicals for endocrine disruption: case studies using example chemicals (No. 181), Organisation for Economic Cooperation and Development, Paris.

Ohlsson, A., Ulleras, E. and Oskarsson, A. (2009) 'A biphasic effect of the fungicide prochloraz on aldosterone, but not cortisol, secretion in human adrenal H295R cells—underlying mechanism.', *Toxicol.Lett.191, pp.174–180*

Ohlsson, A., Cedergreen, N., Oskarsson, A. and Ulleras, E. (2010) 'Mixture effects of imidazole fungicides on cortisol and aldosterone secretion in human adrenocortical H295R cells.', *Toxicology.275, pp.21–28*

Sanderson, J.T., Boerma, J., Lansbergen, G.W.A. and van den Berg, M. (2002) 'Induction and inhibition of aromatase (Cyp19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells.', *Toxicol.Appl.Pharmacol 182, pp.44–54*

Taxvig, C., Vinggaard, A.M., Hass, U., Axelstad, M., Metzdorff, S. and Nellemann, C. (2008) 'Endocrine-disrupting properties in vivo of widely used azole fungicides.', *International Journal of Andrology, 31, pp.170–177.*

Trösken, E.R., Scholz, K., Lutz, R.W., Völkel, W., Zarn, J.A. and Lutz, W.K. (2004) 'Comparative assessment of the inhibition of recombinant human Cyp19 (Aromatase) by azoles used in agriculture and as drugs for humans.', *Endocr.Res.30(3), pp.387–394*

Vinggaard, A.M., Hnida, C., Breinholt, V. and Larsen, J.C. (2000) 'Screening of selected pesticides for inhibition of Cyp19 aromatase activity in vitro.', *Toxicol.In Vitro, 14, pp.227–234*

Vinggaard, A.M., Nellemann, C., Dalgaard, M., Jorgensen, E.B. and Andersen, H.R. (2002) 'Antiaandrogenic effects in vitro and in vivo of the fungicide prochloraz.', *Toxicol.Sci. 69, pp.344–353*

Vinggaard, A.M., Christiansen, S., Laier, P., Poulsen, M.E., Breinholt, V., Jarfelt, K., Jacobsen, H., Dalgaard, M., Nellemann, C. and Hass, U. (2005) 'Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring.', *Toxicol.Sci. 85, pp.886–897*

Winther, C.S., Nielsen, F.K., Hansen, M. and Styrishave, B. (2013) 'Corticosteroid production in H295R cells during exposure to 3endocrine disrupters analyzed with LC-MS/MS.', *Int.J.Toxicol.32(3), pp.219–227*

Prochloraz,

5. Environmental hazard assessment

5.6.2 Endocrine Disruption

5.6.2.1 General approach – environment

The peer reviewed literature was investigated by use of Web of Science including all databases. Search terms included prochloraz + endocrine, prochloraz + fish, prochloraz + amphibian, prochloraz + vitellogenin. A google search including the search terms prochloraz + endocrine, prochloraz + fish, prochloraz + amphibians and prochloraz + vitellogenin were also performed and revealed the OECD case study with prochloraz (OECD GD 181, 2012)

Studies solely investigating effects of prochloraz in invertebrates were not taken into account due to lack of endocrine specific endpoints. Studies using mixtures of chemicals where the effects could not be directly related to prochloraz were also not taken into account. For studies where one of the authors was also an author of this document, the study quality was evaluated by another person.

Four studies included in Table 3 are not evaluated in the present document but were copied from the summary table of OECD GD 181 where they have been evaluated. The studies are Ankley et al., 2005, Biever et al., 2007, Jensen & Ankley, 2006 and OECD, 2006 (ring test results).

Due to the substantial amount of literature on prochloraz and endocrine disruption, only a selection of key studies in relation to identification of endocrine disruption according to the WHO/ICPS definition was evaluated. A search for fish and amphibian studies including endocrine biomarkers and endpoints but showing no endocrine effects of prochloraz was also conducted to investigate the Weight of Evidence of ED-effects of prochloraz in vertebrate wildlife. Searching prochloraz + endocrin* + vitellogenin as well as prochloraz + sex ratio and prochloraz + zebrafish and prochloraz + fathead minnow and prochloraz + Japanese medaka revealed approximately 30 studies in Web of Science related to fish and ED. None of these studies were “negative” on prochloraz ED effects.

5.6.2.2 *In vitro* information indicative of endocrine activity.

Covered by the toxicological evaluation of prochloraz.

5.6.2.3 *In vivo* effects with regard to an endocrine mode of action

Flynn et al. (2017)

Summary: The authors used a Japanese medaka (*Oryzias latipes*) multigeneration assay (modification of OECD TG 240 with much less replication and lower power of endpoints) to investigate effect of prochloraz (5.3, 9.2, 17.5, 25.0 and 41.1 µg/l mean measured) on several endpoints including ED relevant endpoints. The exposure was 29 weeks and spanned 2 full generations. The test was terminated after reproduction in the F2. Six replicates of breeding pairs (one male and one female) per concentration was used where the final OECD TG 240 used 24 pairs for controls and 12 pairs for exposures. Prochloraz did not affect the tissues and organs of male fish, but caused endocrine related pathologies in the ovaries of female fish (decreased yolk and follicular hyperplasia/hypertrophy). The LOEC for fecundity was 41µg/L in F0 and 25 µg/L in F2. The phenotypic sex ratio in prochloraz exposed fish was not discussed, possibly because of low power du to only 12 fish per exposure in F1 and F2. Vtg-gene expression was decreased in females. The authors argue that this could be plausibly

linked to aromatase inhibition, causing circulating E2 to decrease, which in turn would lower the expression of hepatic vitellogenin.

Study quality and assessment: The study is very well described and detailed but due to the test design of only 6 breeding pairs per test concentration the power of the endpoints was low. For example, the power to detect a 30% fecundity decrease was 0.38, which is very far from the desired 0.8. is strong. Based on this the study is assessed to be of moderate quality. Despite low power, the results do though support prochloraz to inhibit aromatase activity by decreasing Vtg-gene expression in females and causing ED related pathologies in the ovaries of female fish. This link is moderate-strong.

Bauman et al. (2015)

Summary: The authors investigated the persistence of effects of prochloraz exposure during sexual development of zebrafish. Fish were exposed (10, 30, 100, and 300 µg/l prochloraz) from fertilization to 60 days post hatch and transferred to clean water for 40 days thereafter. Two replicates of 100 fertilized eggs were used per concentration. Subsampling of fish was performed at day 30 and 60 post hatch and final sampling at 100 days post hatch. No chemical analysis was performed but a reference to Baumann (2012) was given because the same exposure system was used. Baumann et al (2012) is evaluated other place in this document. Survival was not described so it is difficult to assess whether prochloraz affected this. The number of sampled fish are though equal to the number of initial fish so it is expected that mortality was extremely low. Histological investigations of the gonads revealed persistent effects on sexual differentiation. The sex ratio was skewed towards males and significantly more intersex individuals were found and this decrease in phenotypic females was dose dependent with no females at 300 µg/l prochloraz. A 40-day depuration period showed that the masculinization of the fish was irreversible.

Study quality and assessment: The study is very well described and detailed and the results are dose-dependent and expected. It is not explained how survival could be close to 100% and prochloraz was not quantified so the overall quality is assessed to be medium-high. The data on the change in phenotypic sex ratio is population relevant and adverse. The link to aromatase inhibition which is expected for prochloraz in fish is strong.

Bauman et al. (2013)

Summary: Five zebrafish (*Danio rerio*) FSDTs with EDCs with different modes of action including prochloraz (CAS no. 64447-09-5) were conducted, and the experimental setups followed the OECD TG 234. Each tank contained 40 eggs (two replicates), and the prochloraz exposure started at 1 hpf and ended 60 dpf and with the following nominal concentrations: 10, 30, 100, and 300 µg/L. Chemical determination of the actual exposure concentrations was performed by LC-MS (< 20% deviation from the nominal concentration over the entire test period).

No significant effects on hatchability or mortality were observed after exposure to prochloraz, but the body size was reduced in the highest concentrations compared with the control. The results document that not only sex ratio and VTG production of the prochloraz exposed fish were massively affected, but also gonad maturation. The sex ratio was shifted towards males and intersex in the groups exposed to the two highest exposure concentrations; only male and intersex individuals were observed at 300 µg/L and the number of females was significantly reduced at 100 µg/L (≤ 10% females). The VTG response in both males and females was non-monotonic, and the only significant effect was an

increase in male VTG at 30 µg/L. At this concentration, a non-significant increase in VTG was also observed in females, whereas both male and female VTG levels tended to decrease at higher exposure concentrations, albeit not significantly. The maturation index followed the same pattern in both males and females but statistical significant differences were not observed when compared with the control.

Study quality and assessment: The experimental setup follows the OECD TG 234 and the experimental design and laboratory procedures are very well described. However, the authors state that prochloraz did not influence the mortality, but information about the exact mortality in the groups is not mentioned, and n is not provided in any of the figures or figure legends, which complicates evaluation of the data quality. Therefore, the study quality is assessed as medium-high. Clearly, it is demonstrated that prochloraz exposure during sexual development of zebrafish has an adverse effect since the sex ratio is shifted towards males at the two highest exposure concentrations. The only significant increase in VTG is observed in males in the intermediate exposure (30 µg/L). The exact mechanism is unclear but a clear endocrine MoA is demonstrated and the link to an endocrine mediated MoA is assessed as strong.

Holbech et al. (2012)

Summary: Zebrafish or fathead minnow (0-60 dph or 0-120 dph) were exposed to prochloraz (CAS no 67747-09-5) in different laboratories in order to investigate the inter-laboratory robustness of the endocrine parameters in the OECD FSDT TG 234 (whole body VTG and sex ratio) and to compare the sensitivity of the two test species.The experimental setup followed the OECD TG 234 and included two or four replicates with a minimum of 40 eggs per replicate in a flow-through system. The following nominal prochloraz concentrations were used: 32, 100, and 320 µg/l or 38, 75, 150, 300 and 600 µg/L. The exposure concentrations were determined by LC-MS or HPLC-UV. Generally, no significant effects of prochloraz on hatchability and growth was observed, but mortality was affected in a few exposure groups in some of the labs. Prochloraz induced a monotonic concentration-response reduction in VTG levels of female fathead minnows with effect at the lowest (29 µg/L at 60 dph) or intermediate concentration (106 µg/L at 120 dph). In zebrafish, a monotonic concentration-response reduction in female VTG levels was also observed with effects observed at 48, 99 or 183 µg/L, respectively. In two out of three labs a reduction in zebrafish males was also observed with effects at 44 or 135 µg/L. In both fathead minnow experiments the sex ratio was significantly skewed towards males in the highest exposure group (284 and 301 µg/L) but no effect at the intermediate concentration in any of the experiments (≈100 µg/L). The sex ratio of zebrafish was skewed towards males in a monotonic concentration-response relationship in one lab with effects at the lowest concentration (60 µg/L) and in the second lab no effect at the intermediate concentration (48 µg/L) but significantly more males and undifferentiated individuals at 320 µg/L. The third lab had significantly more males at the two intermediate concentrations (99 and 197 µg/L) but not at the highest concentration (434 µg/L), however most ot the water samples from this lab were lost.

Study quality and assessment: The experimental setups are solid with regards to the number of animals, replicates, histological procedures, and chemical analysis of water samples, and the results are well described. However, based on the information in the M&M section and the figures it is unclear how many embryos each laboratory had in each replicate and the exact mortality in each experiment is not mentioned, also the number of control animals is much higher than the number in the exposure group, which is not explained. But n is generally high and it is stated the prochloraz generally did not affect the mortality. The experiment was run with the same setup and in different labs and with different strains of fish, and thereby a broad picture of the effects is obtained. The

study is assessed to be of high quality. The reduction in female VTG and number of females (adverse effect) clearly demonstrate an endocrine MoA. Prochloraz has multiple endocrine modes of action, including inhibition of aromatase activity, inhibition of androgen synthesis and AR antagonism, in fish and mammals, and based on these experiments it is not clear if the exact mechanism is anti-estrogenic, androgenic or inhibition of aromatase activity. However, the evidence for an ED MoA is assessed as strong.

Thorpe et al. (2011)

Summary: In the present study, fathead minnow (*pimephales promelas*) and zebrafish (*Danio rerio*) were exposed to prochloraz (32, 100 and 320 µg/l) from embryo until 95-125 days post hatch (DPH) and 60 DPH respectively. The exposure periods cover the sexual development of the two fish species. 2x25 embryos per replicate in 6 replicates for fathead minnow and 2x30 embryos per replicate in 4 replicates for zebrafish were used until 30 DPH where each replicate was adjusted to 30 fish by random removing excess fish to equalize fish density. Exposure to 320 µg/L inhibited somatic growth in both species. Larval survival in zebrafish was decreased from 32 µg/l where such effect was not seen in fathead minnow. Prochloraz exposure caused a decrease in the proportion of phenotypic females in both species with a zebrafish LOEC of 100 µg/l and a fathead minnow LOEC of 320 µg/L. A delay in the completion of sexual differentiation was also seen in both species.

Study quality and assessment: The study is very well described and detailed and replication is high. The quality is high. The data on the change in phenotypic sex ratio is population relevant and adverse. The link to aromatase inhibition is strong due to the decreased VTG concentration in female fathead minnow and zebrafish.

Brande-Lavridsen et al. (2008)

Summary: In this amphibian study, tadpoles of the Common frog (*Rana temporaria*) were exposed to prochloraz or EE2 from hatch through metamorphosis. In two different experiments, tadpoles were exposed to 15 and 150 µg/l prochloraz (nominal concentrations) and 11, 115 and 252 µg/l prochloraz respectively. Two replicates of 150 tadpoles were used per concentration. Exposure was semi-static with water exchange 3 times per week. Tadpoles were sampled at the end of metamorphic climax (Gosner stage 44–46). Testosterone and E2 was quantified in whole body homogenate by commercial ELISA-kits. Calcium levels was measured in whole body homogenate as a surrogate for vitellogenin. Gonadal histology was performed to investigate effects on gonads and to document phenotypic sex. Survival rates ranged between 71.3 and 84.3%. The sex ratio was significantly skewed toward males from 115 µg/l prochloraz and above and testosterone concentrations decreased in the same groups. E2 increased in the 115 µg/l group but not at the highest exposure concentration of 252 µg/l. Calcium was not affected by prochloraz.

Study quality and assessment: The study is well described. The use of calcium as surrogate for vitellogenin is not recommendable so these results are not taken into account. The study is assessed to be of moderate-high quality. The results on the phenotypic sex ratio is population relevant and adverse and highly linked to an endocrine mechanism. The reduced T concentrations could be caused by different mechanisms but is not directly linked to aromatase inhibition.

Kinnberg et al. (2007)
Summary: The objective of this study was to assess the effects of prochloraz on the sexual development of zebrafish (*Danio rerio*) exposed to prochloraz (Information about purity and CAS no. not provided) for 60 days from 24 h post fertilization (0, 16, 64 or 202 µg/l). The experimental setup followed the OECD TG 234 and included two replicates per exposure with 80 eggs per replicate in a flow-thorugh system. The exposure concentrations were determined by LC-MS. The sex ratio was significantly skewed towards males in the highest exposure group and this group also had a significantly higher number of intersex individuals. Male VTG concentrations were significantly increased at the low and intermediate prochloraz concentration and at the highest concentration both male and female VTG concentrations were significantly decreased.

By histological examination of the gonad development it was shown that females in all exposure had gonads with less developed oocytes compared with the control, and when the males of all prochloraz treatments were pooled 82% of the males had gonads in the most developed stage (stage III: abundant spermatozoa) compared with 66% in the control group. By inhibition of the aromatase activity 17β - estradiol synthesis is reduced and a decrease in female VTG and female individuals would be expected. The observed decrease in male and female VTG levels at the highest concentration is consistent with aromatase inhibition, and the enhanced sperm production in males and increased number of males could also be related to an increased androgen concentration or decreased E2/T ratio. The increase in male VTG concentration at the low and intermediate concentration may be due to the ability of prochloraz to target multiple steroidogenic enzymes, thus leading to effects elsewhere in the steroidogenic pathway.

Study quality and assessment: The experimental setup is solid with regards to the number of animals, replicates, histological procedures, and chemical analysis of water samples, and the results are well described. The mortality is approximately 50% but not different between the exposure groups and the control. The study is assessed to be of high quality. The results on the phenotypic sex ratio is population relevant and adverse. The link to aromatase inhibition is strong due to the decreased VTG concentration in female zebrafish but other endocrine MoAs cannot be ruled out.

Additional information used:
OECD Case study with prochloraz OECD GD 181 (http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)34&doclanguage=en).

5.6.2.4 Summary of the plausible link between adverse effects and endocrine mode of action

The evidence from the referred studies strongly support that prochloraz is an endocrine disrupter according to the IPCS/WHO definition (summarized in Table 3). It was not possible to find any study with fish that was investigating phenotypic sex ratio without finding an effect of prochloraz exposure.

5.6.2.5 Environmental relevance

Adverse effects (Table 4) in fish and amphibians are seen around and below 100 µg/l. Barret (1995) calculated the maximal predicted environmental concentration (PEC) to 195 µg/l. The PEC was based on an overspray of water 30 cm deep at a maximum recommended application rate of 0.585 kg active ingredient ha^{-1}. An EFSA risk assessment of prochloraz did though conclude a low risk of prochloraz to aquatic organisms (EFSA, 2011).

Table 3 Overview of *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of prochloraz in fish and amphibians

Reference	**MoA**		**Quality of study**	**Evidence for ED MoA**
	In vitro	*In vivo*		
Flynn et al. (2017)		Japanese medaka multi-generation test. Dosing 5-40 µg/l prochloraz. Decreased fecundity. Decreased Vtg-gene expression. ED related pathologies in the ovaries of female fish (decreased yolk and follicular hyperplasia/hypertrophy).	medium	Moderate-strong
Baumann et al. (2015)		Zebrafish exposed from 0-60 dpf. Depuration period from 60-100 dpf. Skewed phenotypic sex ratio. NOEC 30 µg/l. LOEC 100 µg/l (no females at 300 µg/l.	Medium-high	Strong
Baumann et al. (2013)		Zebrafish exposed from 0-60 dpf. The sex ratio was shifted towards males and intersex in the groups exposed to the two highest exposure concentrations; only male and intersex individuals were observed at 300 µg/L and the number of females was significantly reduced at 100 µg/L	High	Strong
Holbech et al. (2012)		Zebrafish exposed from 0-60 dpf. Fathead minnow exposed from 0-60/120 dpf. Dose-range 32-600 µg/l. Five studies were conducted as a part of the OECD TG 234 validation. Effects on sex ratio (decreased proportion of females) and Vtg protein levels were seen in all experiments.	High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	In vitro	*In vivo*		
Thorpe et al. (2011)		Zebrafish exposed from 0-60 dph. Fathead minnow exposed from 0-95 or 125 dph. Dosing: 32, 100 and 320 µg/l. Phenotypic sex ratio skewed with less females. LOEC zebrafish, 100 µg/l. LOEC fathead minnow, 320 µg/l. Delayed completion of sexual differentiation in both species.	High	Strong
Brande-Lavridsen et al. (2008)		Common frog Rana temporalis tadpoles exposed from hatch through metamorphosis. The sex ratio was significantly skewed toward males from 115 µg/l prochloraz and above and T decreased in the same groups. E2 increased in the 115 µg/l group but not at the highest exposure concentration of 252 µg/l.	Moderate-high	Strong
Ankley et al. (2005)		From OECD GD 181: OECD TG 229: Fish Short Term Reproduction Assay (FSTRA): Dosing range = 30 -300 µg/L Fecundity ↓ >3-fold in fathead minnow (LOEC = 100 µg/L; NOEC = 30 µg/L). ♀VTG↓ approx. 3-fold in (LOEC = 100 µg/L; NOEC =30 µg/L). Significant reductions were also observed in ♂ testosterone, 11ketotestosterone and brain aromatase activity, and ♀ estradiol.	High	Strong
Biever et al. (2007)		From OECD GD 181: OECD TG 229: Fish Short Term Reproduction Assay (FSTRA): Dosing range = 20 -300 µg/L Fecundity↓ approx. 5-fold in fathead minnow (geomean LOEC = 300 µg/L; geomean NOEC = 100 µg/L).	High	strong

Reference	MoA		Quality of study	Evidence for ED MoA
	In vitro	*In vivo*		
Kinnberg et al. (2007)		Zebrafish exposed from 0-60 dpf. Dose-range 16-202 µg/l (measured). Effects on sex ratio (decreased proportion of females) and Vtg protein levels were seen. NOEC sex ratio 64 µg/l, LOEC 202 µg/l	High	strong
Jensen & Ankley (2006)		From OECD GD 181: Three individual studies following OECD TG 229: Fish Short Term Reproduction Assay (FSTRA): Dosing range = 20 -300 µg/L Fecundity↓ in fathead minnow (geomean LOEC = 58 µg/L; geomean NOEC = 16 µg/L). Secondary sexual characteristics (tubercle score) ↓ in fathead minnow (geomean LOEC = 144 µg/L; geomean NOEC = 34 µg/L). ♀VTG↓ in fathead minnow (LOEC = 20 µg/L; NOEC = <20 µg/L)	High	Strong
OECD (2006)		From OECD GD 181: Ring test of OECD TG 230 with fathead minnow, Japanese medaka and zebrafish: Dosing range = 20 - 300 µg/L ♀VTG↓ by up to 10-fold in medaka (geomean LOEC = 116 µg/L; geomean NOEC = 38 µg/L), fatheads (geomean LOEC = 208 µg/L; geomean NOEC = 58 µg/L), and zebrafish (geomean LOEC = 182 µg/L; geomean NOEC = 49 µg/L)2 Fecundity was reduced in medaka (4/4 labs) and zebrafish (1/2 labs).	High	Strong

Table 4. Overview of potential endocrine-related adverse effects of prochloraz in fish and amphibians.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Baumann et al (2015)	Zebrafish	Skewed phenotypic sex ratio. No females at 300 µg/l (nominal)	Medium-high	high
Baumann et al (2013)	Zebrafish	Skewed phenotypic sex ratio.	Medium-high	high
Holbech et al (2012)	Zebrafish	Skewed phenotypic sex ratio.	high	high
Holbech et al (2012)	Fathead minnow	Skewed phenotypic sex ratio.	high	high
Thorpe et al (2011)	Zebrafish	Skewed phenotypic sex ratio.	high	high
Thorpe et al (2011)	Fathead minnow	Skewed phenotypic sex ratio.	high	high
Brande-Lavridsen et al (2008)	Rana temporalis	Skewed phenotypic sex ratio. No females at 115 µg/l and above (measured)	high	high
Kinnberg et al (2007)	zebrafish	Skewed phenotypic sex ratio.	high	high

References

Ankley, G.T., Jensen, K.M., Durhan, E.J., Makynen, E.A., Butterworth, B.C., Kahl, M.D., Villeneuve, D.L., Linnum, A., Gray, L.E., Cardon, M., Wilson, V.S., (2005).' Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (Pimephales promelas)'. *Toxicological Sciences 86, 300-308.*

Barrett, K.L. 1995. A comparison of the fate and effects of prochloraz in artificial and natural sediments. Journal of Aquatic Ecosystem Health 4: 239-248.

Baumann, L., Holbech, H., Keiter, S., Kinnberg, K.L., Knoerr, S., Nagel, T., Braunbeck, T.,(2013). 'The maturity index as a tool to facilitate the interpretation of changes in vitellogenin production and sex ratio in the Fish Sexual Development Test.' *Aquatic Toxicology 128, 34-42.*

Baumann, L., Knorr, S., Keiter, S., Nagel, T., Segner, H., Braunbeck, T., (2015). 'Prochloraz causes irreversible masculinization of zebrafish (Danio rerio).' *Environ. Sci. Pollut. Res. 22, 16417-16422.*

Biever, R.C., Kruger, H., Kern, M., Blackshear, P.E., Sloan, C.S., (2007). 'Inter-laboratory validation of the fish short-term reproduction assay run simultaneously across three independent contract laboratories'. Draft final report to the US EPA. EPA Contract no. EP-W-06-026, 96 pp.

Brande-Lavridsen, N., Christensen-Dalsgaard, J., Korsgaard, B., (2008). 'Effects of prochloraz and ethinylestradiol on sexual development in Rana temporaria. Journal of Experimental Zoology Part a'- *Ecological Genetics and Physiology 309A, 389-398.*

EFSA Journal 2011; 9(7):2323. Conclusion on the peer review of the pesticide risk assessment of the active substance prochloraz. European Food Safety Authority, Parma, Italy.

Flynn, K., Lothenbach, D., Whiteman, F., Hammermeister, D., Touart, L.W., Swintek, J., Tatarazako, N., Onishi, Y., Iguchi, T., Johnson, R., (2017). 'Summary of the development the US Environmental Protection Agency's Medaka Extended One Generation Reproduction Test (MEOGRT) using data from 9 multigenerational medaka tests'. *Environ Toxicol Chem 36, 3387-3403.*

Holbech, H., Kinnberg, K.L., Brande-Lavridsen, N., Bjerregaard, P., Petersen, G.I., Norrgren, L., Orn, S., Braunbeck, T., Baumann, L., Bomke, C., Dorgerloh, M., Bruns, E., Ruehl-Fehlert, C., Green, J.W., Springer, T.A., Gourmelon, A., (2012). 'Comparison of zebrafish (Danio rerio) and fathead minnow (Pimephales promelas) as test species in the Fish Sexual Development Test (FSDT).' *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 155, 407-415.*

Jensen, K.M., Ankley, G.T., (2006). 'Evaluation of a commercial kit for measuring vitellogenin in the fathead minnow (Pimephales promelas)'. *Ecotoxicology and Environmental Safety 64, 101-105.*

Kinnberg, K., Holbech, H., Petersen, G.I., Bjerregaard, P., (2007). 'Effects of the fungicide prochloraz on the sexual development of zebrafish (Danio rerio)'. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 145, 165-170.*

OECD, (2006).' OECD Final Draft Report of Phase 1B of the Validation of the 21-day Fish Assay for Detection of Endocrine Active Substances'. *131 pp.*

OECD, (2012). OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 181. Guidance document (GD) on standardised test guidelines for evaluating chemicals for endocrine disruption: Case studies using example chemicals. .

Thorpe, K.L., Pereira, M.L.A.M., Schiffer, H., Burkhardt-Holm, P., Weber, K., Wheeler, J.R., (2011). 'Mode of sexual differentiation and its influence on the relative sensitivity of the fathead minnow and zebrafish in the fish sexual development test.' *Aquatic Toxicology 105, 412-420.*