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GUIDANCE DOCUMENT (GD) ON STANDARDISED TEST GUIDELINES FOR EVALUATING CHEMICALS FOR ENDOCRINE DISRUPTION: CASE STUDIES USING EXAMPLE CHEMICALS

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IOMC INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

FOREWORD

This document includes case studies developed to evaluate whether the conclusions and next steps recommended in the Guidance Document (GD) on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (published as No. 150 in the Series on Testing and Assessment) are sensible and helpful when assessed in light of comprehensive datasets. Three case studies (for Prochloraz, 4-tert-Octylphenol, and Perchlorate) were developed by two consultants in close consultation with the Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA AG). The first case study was discussed at a meeting of the EDTA AG in April 2011. The three case studies were then discussed at a second meeting of the EDTA AG in December 2011. In November 2011, comments were requested from the Working Group of National Coordinators of the Test Guidelines Programme (WNT). The document was revised on the basis of the comments received at the EDTA AG meetings and from the WNT. It was then submitted to the WNT for approval at its meeting held on 24-27 April 2012. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (hereafter Joint Meeting) agreed to its declassification on 26 July 2012.

This document is published under the responsibility of the Joint Meeting.

INTRODUCTION

1. The OECD Endocrine Disruption Testing and Assessement Advisory Group (EDTA AG) has developed a Guidance Document (GD) on "Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption" (OECD, 2012). During the writing of the GD it was decided that a series of case studies of known endocrine active substances should be conducted to test the guidance given in the draft GD. The objectives of the draft GD are:

- To support regulatory authorities' decisions on the hazard of specific chemicals and toxicologically-relevant metabolites when they receive test results from a Test Guideline (TG), draft TG, or other standardised assay for the screening/testing of chemicals for endocrine disrupting properties. The contexts for these decisions will vary, depending on local legislation and practice. The advice is therefore worded in such a way as to permit flexible interpretation.

- To provide guidance on how to interpret the outcome of individual assays and how to increase evidence on whether or not a substance may be an ED. In the context of the GD, an endocrine disrupter (ED) has been defined according to WHO (2002) (see Glossary). Testing strategies or guidance on interpretation from a suite of tests are not given.

- Hazard assessment methods in the guidance are arranged in a two step process:

    - Use of a harmonised framework for assessing assay results together with existing information on likely or known hazards should avoid unnecessary animal testing.

    - Recommendation of a test method that may be performed if regulatory authorities need more evidence.

2. The purpose of these case studies, which should always be considered together with the GD, is to evaluate whether the Conclusions and Next Steps recommended in the GD are sensible and helpful when assessed in the light of comprehensive datasets. Such an assessment will hopefully provide greater confidence in the advice contained in the GD. The case studies may also lead to amendments being made to the GD as it is intended to be a "living document" that will be updated as science in this field develops.

It is important to note that the case studies are intended to challenge the guidance given about the standardized assays and not to provide an assessment of the chemicals themselves, and to that extent these studies are hypothetical, even though they use real data. The case studies (which only address the interpretation of data obtained using OECD test guidelines) must, under no circumstances, be used to reach conclusions about the environmental and human hazards and risks which might be posed by these chemicals, because the literature has not been exhaustively reviewed, the scientific quality of the papers has not be formally assessed, and some papers have only been seen in summary. In other words, the evaluations in this document must not be considered as making definitive conclusions about whether or not the substances in question are endocrine disrupting chemicals.

3. Informal discussions were held by the consultants and various EDTA AG members in early 2011 about possible candidate chemicals suitable for case studies. It was considered desirable that the case studies should cover the endocrine modalities addressed by the GD *i.e.*

- Estrogen receptor mediated
- Androgen receptor mediated
- Thyroid hormone mediated

- Steroidogenesis interference

4. In order to adequately assess the guidance given for each of the assays, it is important that a reasonably comprehensive dataset should be available for the chemicals. The dataset should cover a range of *in vitro* and *in vivo* screens (mammalian and wildlife), as well as higher tier *in vivo* studies, drawn from all levels of the OECD Conceptual Framework (CF). In particular, single- or multiple-lifecycle test data were considered essential, because only these could provide a reasonably reliable standard against which the results of lower level assays could be compared. It should be noted that the reasons why a particular scenario is evaluated for a given assay outcome are briefly set out at the start of each evaluation. Scientific quality of the key studies underpinning the choice of scenario is considered to be acceptable, although the quality of papers has not been formally evaluated.

5. The requirement for lifecycle data strongly limits the numbers of chemicals that would be suitable for case studies, and there are no chemicals for which endocrine-relevant test data are available from the full range of possible standardised assays. The chemicals chosen were selected because they had large databases available, covered multiple modes of action and were representative of different classes of chemicals *e.g.* pesticides and HPV chemicals. Data on toxicity to humans has not been considered and the potential exposure of humans and wildlife populations to these chemicals has also not been analysed here as these areas were not included in the GD. Although a weight of evidence assessment of the chemicals themselves should consider these factors, they are not relevant for these case studies for evaluation of the GD.

2.Chemicals Selected

6. The chemicals selected for the case study are listed below:

- Prochloraz is an imidazole fungicide. Its fungicidal activity is due to inhibition of 14 alpha-demethylase (CYP 51), an enzyme required for synthesis of fungal cell walls. It has multiple mechanisms of action in non-target species including inhibition of enzymes of steroidogenesis (CYP 19, CYP 17 and 5 alpha-reductase) and AR antagonism. Other possible mechanisms of action are ER antagonism and AhR agonism.

- 4-Tert-octylphenol (tert-OP) is a member of the general chemical family of alkylphenols. It is a chemical intermediate, and is mainly (>97%) used to make phenolic resins. A small percentage of tert-OP is converted into octylphenol ethoxylate surfactants. Resins and ethoxylates made from tert-OP are used in a variety of industrial processes. Tert-OP occurs *inter alia* in surface waters as a result of degradation and from a number of industrial processes. It is a weak estrogen mimic acting as an ER agonist.

- Perchlorate is an anion associated with several cations including ammonium, potassium and sodium. It is used primarily as an oxidising agent in some rocket fuels and can be discharged to surface waters from manufacturing plants. However, it is also formed naturally in the environment. It is an inhibitor of the sodium iodide symporter (NIS), and competitively inhibits the uptake of iodide by the thyroid gland. This in turn can lead to a reduction in the synthesis of thyroid hormones (T3 and T4) as their molecules contain 3 and 4 atoms of iodine, respectively.

3. Approach

3.1 Collection of Data

7. Data on prochloraz, octylphenol and perchlorate were gathered from publicly available sources (OECD, USEPA, Pubmed, Web of Science) covering both internationally standardised OECD TGs and non-standard methods. Only data relevant for assessing the standardized methods included in the GD were used,

although this included most data relating to endocrine effects. The modalities under consideration are primarily EATS but other data are also considered. As the objective of the case studies was assessment of the guidance for the TGs and not the chemicals themselves, no judgement was made on the quality of studies and in some cases the original reports have not been obtained.

8. Results for assays corresponding to those given in the GD are listed in Tables 4.1.1, 5.1.1 and 6.1.1. The structure of the tables follows the Contents Table of the GD so that the two documents can easily be used together. Results listed under "Standard Assay" are those where the method described is considered to be very similar, or identical, to that given in the appropriate TG. Results listed under "Non-standard Assay" are those where the method described is also considered to be scientifically very similar to that given in the appropriate TG but with significant methodological differences. Qualitative data may be compared between "Standard" and "Non-standard" assays but quantitative comparisons may not be valid, *e.g.* tests should be consistently positive or negative but IC50s or NOAELs may vary.

9. Data listed under in Tables 4.1.2, 5.1.2 and 6.1.2 do not comply with the study designs in the CF but are considered to be of relevance for assessment of endocrine effects on the health of humans or wildlife. *In vitro* and *in vivo* data are given separately (under the headings in the "Building Blocks" within the GD) and are listed in author alphabetical order.

3.2 Use of Case-Study Data in Building Blocks and Scenarios

10. The methods used for conducting the case study are straightforward. Each assay for which guidance is given in the draft GD has been evaluated using the relevant data inserted into the building block in the draft GD. We have tried to mimic real-life situations. For any given assay, 'existing data' have been assumed to be those which are likely to be available for a chemical that is both a "possible ED" (see glossary) and a pesticide or HPV chemical. Thus, for example, we have assumed that data on effects in wildlife are unlikely to be available when assessing the results of *in vitro* assays. Existing data will vary according to chemical, *e.g.* a pesticide like prochloraz will have had many tests conducted whilst HPV chemicals will have had a more limited number conducted. In all cases *ad-hoc* literature studies may be available and these are also included as "existing data". *In vitro* data are assumed to be available in all cases but it is recognised that this will not always be so.

11. We have also assumed that in most cases, an assay from the lower levels of the CF is unlikely to be run if substantial higher level data are available (with the exception of the *in vitro* assays in Level 2). At each level of the CF, we have therefore assumed that the "existing data" comprise a range of *in vitro* data ("*in vitro* mechanistic data"), plus general *in vivo* data from non-endocrine specific tests, *ad hoc* literature data and *in vivo* data limited to the CF Level under consideration and below ("*in vivo* data of concern"). Furthermore, we have not used QSAR-predictions or read-across from data on similar chemicals, as should be done if one were evaluating a chemical in the real world and as detailed in the GD itself.

12. It would have been possible to conduct evaluations with artificially limited sets of 'existing data' (*e.g.* to assume that no or few other endocrine assay data are available), but again, this has not been done as we wished to keep artificial manipulation to a minimum. Clearly, the confidence that can be derived from a chemical with a large amount of data would have to be downgraded in cases of chemicals with more limited data.

13. In each case, we have made an assessment about whether, a) the 'Possible Conclusions' in the relevant building block for the assay, and b) the possible 'Next Steps', are reasonable in the light of the other data available to us, and especially in view of the data from higher levels in the CF. It should be noted that our assessments of how the GD addresses each assay have been made solely in the light of data available to the authors. No weight-of-evidence assessment of the chemicals has been made, and non-endocrine mechanisms of toxicity have not been considered here. On the other hand, the GD stresses the importance

of considering all data when assessing the endocrine disrupting potential of a chemical. This should include conducting a weight of evidence assessment of assay results, QSAR and read-across data, human data and an assessment of exposure potential.

3.3 Presentation of results of the case studies

14. The results of the case studies are set out in tabular form, following the order used in the GD, *i.e. in vitro* assays, followed by the *in vivo* wildlife assays and lastly the *in vivo* mammalian assays.

15. Four sub-sections are given for assay. These comprise:

1) A brief summary of the available data.

2) A summary of the Conclusions, Next Step(s) as suggested in the building block and outcome of that Step when the chemical is tested. The wording in the table mirrors that used in the building block.

3) A list of the Other Considerations and the conclusions that can be derived from them using the available data for the chemical.

4) The Overall Conclusions from the assessment.

4. Prochloraz Case Study Results

4.1 Data used in the Prochloraz Case Study Analysis

16. Data for standardised assays used in the case study on prochloraz for GD No. 150 are given in Table 4.1.1. The format of the table follows that given in the Contents of the GD so that the two documents can be easily compared. Data, from non-standardised assays, considered relevant for assessment of endocrine effects are given in Table 4.1.2. These data do not comply with the Test Guideline study designs in the CF but are considered to be of relevance for assessment of effects on the health of humans or wildlife.

Table 4.1.1: Data for standardised assays used in the case study on prochloraz for The OECD GD on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (No. GD 150).

Note that the "Result" column indicates a positive (+), negative (-) or equivocal (Eq) result for endocrine endpoints only.

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
	A. Validated assays for which guidance is provided in the main GD			
	***In vitro* Screens**			
C.2.1	ER Binding Assay (US EPA OPPTS 890.1250)		No data available	
C.2.2	AR Binding Assay (US EPA OPPTS 890.1150)	+	**Standard Assay:** Rat prostate extract. Prochloraz displaced R1881 with IC50 of 60µM.	Noriega *et al* (2005)
		+	**Non-standard Assay:** Rat recombinant fusion protein. Prochloraz displaced R1881 with IC50 of 40.6µM. RBA was 0.0126% (*c.f.* 100% for DHT).	Freyberger *et al* (2010a)
C.2.3	OECD TG 455: The Stably Transfected Human ERα Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA) (including Guidance for the Antagonism Assay)		**Standard Assay:** No data available	
		+	**Non-standard Assay:** Transient transfection assay (MCF-7 cells) with luciferase as reporter gene. Prochloraz was tested in the presence and absence of E2. No agonism detected but antagonism was evident with LOEC of 10 µM and 51% inhibition of the maximum response with E2.	Andersen *et al* (2002)
		+	Assay system as above but including stably transfected MVLN cells (in addition to transiently transfected MCF-7 cells). No agonism detected but antagonism was evident with LOEC of 25 or 10 µM for MVLN or MCF-7 cells respectively.	Bonefeld-Jorgensen *et al* (2005)
C.2.4	OECD TG 456: H295R	+	**Standard Assay:** Dose-dependent decrease in T and E2 at	Hecker *et al*

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
	Steroidogenesis Assay		prochloraz concentrations greater than 0.03 and 0.003 µM respectively. A maximum effect was obtained at 1.0 µM where E2 was 5.5-fold and T 3-fold less than the solvent control. [P was increased].	(2006)
		+	Dose-dependent decrease in T and E2 with prochloraz. Effects at 0.01µM (lowest concentration tested). Maximum effects (at 3.0 µM) were less than 10% of the solvent control. [P was increased].	Kjaerstad *et al* (2010)
		+	Dose-dependent decrease in T and E2 with prochloraz. Effects at 0.01 µM (lowest concentration tested) for T and 0.1µM for E. [P was increased].At the highest concentrations (1 &3 µM) T was almost completely inhibited.	Laier *et al* (2006)
		+	Validation of H295R assay by 4 labs. Prochloraz produced a dose-dependent decrease in T and E2 in all cases. Maximum changes observed were generally >0.01-fold change for T and 0.5 to 0.25-fold change for E2. EC50s were T: 0.0099, 0.057, 0.0075, 0.028, >0.01 µM (mean 0.022 µM) and E2: 0.068, 0.27, 0.040, >0.1, >0.038 µM (mean 0.103 µM).	OECD (2010)
		+	T and E2 production were decreased, dose-dependently, at prochloraz concentrations greater than 0.1 and 0.01 µM respectively.	Villeneuve *et al* (2007)
		+	**Non-standard Assay:** Fathead minnow ovary explants were used. T and E2 production were decreased, dose-dependently, at prochloraz concentrations greater than 7.4 and 2.5 µM respectively.	Villeneuve *et al* (2007)
C.2.5	Aromatase Assay (US EPA OPPTS 890.1200)	+	**Standard Assay:** Validation of human placental and recombinant aromatase assays by 4 labs. Prochloraz inhibited aromatase in all cases. IC50s for human placental assay	USEPA (2007a)

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
			were: 0.0202, 0.0269, 0.0408, 0.0258 µM (mean 0.0284 µM). IC50s for recombinant assay were: 0.0334, 0.0325, 0.0198, 0.0284 µM (mean 0.0285 µM).	
		+	Aromatase was inhibited by prochloraz (human placental microsomes as enzyme source). IC50 was 0.34 µM).	Vinggaard *et al* (2000).
		+	**Non-standard Assay:** Aromatase inhibition tested at a single concentration (50 µM) using human placental microsomes. Activity in presence of prochloraz was 10% of control value (no inhibition being 100%).	Andersen *et al* (2002)
		+	Aromatase inhibition determined using H295R cells. Ki for prochloraz was 0.04 µM.	Heneweer *et al* (2004)
		+	Aromatase inhibition determined in JEG-3 cells. IC50 for prochloraz was less than 1µM.	Laville *et al* (2006)
		+	Aromatase inhibition determined using H295R cells. Ki for prochloraz was 0.037 µM and IC50 0.1 µM.	Sanderson *et al* (2002)
		+	Aromatase inhibition determined using recombinant human enzyme with artificial substrate. IC50 for prochloraz was 0.047 µM.	Trosken *et al* (2004)
		+	Aromatase inhibition determined (as above) using recombinant human enzyme but with T as substrate. IC50 for prochloraz was 0.44 µM.	Trosken *et al* (2006)
	Wildlife Screens and Tests			
C.3.1	OECD TG 229: Fish Short Term Reproduction Assay (FSTRA)	+	**Standard assay:** 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 fathead minnow (LOEC = 100 µg/L; NOEC =	Ankley *et al*. (2005)

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
			30 µg/L). NB: Significant reductions were also observed in ♂ testosterone, 11-ketotestosterone and brain aromatase activity, and ♀ estradiol.	
		+	Dosing range = 20 -300 µg/L **Fecundity**↓ approx. 5-fold in fathead minnow (geomean LOEC = 300 µg/L; geomean NOEC = 100 µg/L).	Biever *et al*. (2007)
		+	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)[1] ♀**VTG**↓ in fathead minnow (LOEC = 20 µg/L; NOEC = <20 µg/L)	Jensen and Ankley (2006)
C.3.2	OECD TG 230: 21 Day Fish Assay	+	**Standard assay:** 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] NB: In this ring test, **fecundity** was also reduced in medaka (4/4 labs) and zebrafish (1/2 labs)	OECD (2006)

[1] Approximate geometric mean values (based on nominal concentrations) are given where data are available from more than one lab in a ring test. These values are only approximate as some NOECs or LOECs were unbounded.

[2] Approximate geometric mean values (based on nominal concentrations) are given where data are available from more than one lab in a ring test. These values are only approximate as some NOECs or LOECs were unbounded.

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
C.3.3	OECD GD 140: Variant of OECD TG 230: Androgenised Female Stickleback Screen (AFSS)		No data available	Katsiadaki pers. comm. 2011
C.3.4	OECD TG 234: Fish Sexual Development Test (FSDT)	+	**Standard assay:** Dosing range = 16-202 µg/L **Proportion of** ♂ ↓ by 5-fold in zebrafish (LOEC = 202 µg/L; NOEC = 64 µg/L). ♂ **and** ♀**VTG**↓ by >100-fold in zebrafish (LOEC = 202 µg/L; NOEC = 64 µg/L). NB: ♂ **VTG** in zebrafish was slightly elevated at 16 and 64 µg/L.	Kinnberg *et al*. (2007)
		+	Dosing range = 32-320 µg/L **Proportion** ♀ ↓ approx. 2-fold in zebrafish (geomean LOEC = 138 µg/L; geomean NOEC = 44 µg/L). Note that one lab reported a LOEC of 22 µg/L and an unbounded NOEC of <22 µg/L, but the dose-response was non-monotonic. Fathead minnow proportion ♀ ↓ by up to 8-fold (geomean LOEC = 292 µg/L; geomean NOEC = 101 µg/L). ♀**VTG**↓ by up to 1000-fold in zebrafish (geomean LOEC = 124 µg/L; geomean NOEC = 40 µg/L) and up to 1000-fold in fathead minnow (geomean LOEC = 30 µg/L; geomean NOEC = <30 µg/L). ♀**VTG**↓ by 4-fold in three-spined stickleback (LOEC and NOEC <32 µg/L). **Proportion** ♀ stickleback not altered (LOEC >320 µg/L)	OECD (2007); Holbech *et al*., 2011 Katsiadaki *et al*. – unpublished data
C.3.5	Fish Lifecycle Toxicity Test (FLCTT) (USEPA OPPTS 850.1500)		No data available	
C.3.6	OECD TG 231: Amphibian		No data available	

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
	Metamorphosis Assay (AMA)			
C.3.7	OECD TG 206: Avian Reproduction Test		No data available	
	Mammalian Screens and Tests			
C.4.1	OECD TG 440: Uterotrophic Bioassay in Rodents (UT assay) (Including GD on the Use of the Assay to Screen for Anti-Estrogenicity)		No data available	
C.4.2	OECD TG 441: Hershberger Bioassay in Rats (H Assay) (Including OECD GD for Weanling Hershberger Bioassay)		**Standard Assay:**	
		+	Castrated rat assay for antagonism. Prochloraz given by gavage at 0, 15.6, 31.2, 62.5 or 125 mg/kg/day for 10 days. Two (of 5) SAT weights were reduced at 125 mg/kg/day (n=6).	Blystone *et al* (2007)
		+	Castrated rat assay for antagonism. Two experiments with prochloraz [1: single dose (250 mg/kg/day); 2: three doses (50, 100 & 200 mg/kg/day). SATs were reduced at all doses. T4 & TSH also reduced (n=6).	Vinggaard *et al* (2002)
		+	Castrated rat assay for antagonism. Prochloraz given orally at 100 mg/kg/day for 7 days. All SATs reduced. T4 also reduced (n=6).	Vinggaard *et al* (2005a)
			Non-standard Assay:	
		-	Castrated rat assay for antagonism (single concentration). No effect at 25 mg/kg/day (n=6).	Birkhoj *et al* (2004)
C.4.3	Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats (Male PP Assay) (US EPA OPPTS 890.1500)		**Standard Assay:**	
		+	Rats given prochloraz by gavage at doses of 0, 31, 62.5 or 125 mg/kg/day from PND 23 to 42 or 51. Age at PPS was increased at 125 mg/kg. SAT weights reduced at 62.5 & 125 mg/kg/day. Serum T was reduced whilst P & hydroxy-P increased at all doses. No NOAEL established (n=8-10).	Blystone *et al* (2007)

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
		+	**Non-standard Assay:** Rats given prochloraz by gavage at doses of 0, 3.9, 7.8, 15.6, 31.3 or 62.5 mg/kg/day from PND 23 to 42. No reduction in SAT weights. Serum T was reduced from 15.6 mg/kg/day whilst progesterone & hydroxy-P were unaffected. NOAEL was 7.8 mg/kg/day based on serum T (n=6).	Blystone *et al* (2007)
C.4.4	Pubertal Development and Thyroid Function Assay in Peripubertal Female Rats (Female PP Assay) (US EPA OPPTS 890.1450)		No data available	
C.4.5	OECD TG 407: Repeated Dose 28 Day Oral Toxicity Study in Rodents	+	**Standard Assay:** No data available **Non-standard Assay:** Male and female Wistar rats were given prochloraz by gavage at doses of 0, 25, 100 or 400 mg/kg/day for 30 days (n=5). Some signs of systemic toxicity occurred at 400 mg/kg/ day, *e.g.* salivation and reduction in weight gain. Liver weight was increased in both sexes at 100 and 400 mg/kg/day. The weight of the adrenals was increased, and the prostate and seminal vesicles were small in the males at 100 and 400 mg/kg/day but no microscopic findings were reported.	Lancaster & Shaw (1980) (Reported in JMPR, 2001)
C.4.6	OECD TG 416: Two-Generation Reproduction Toxicity Study (Including TG 415: One-Generation Reproduction Toxicity Study)	-	**Standard Assay:** Prochloraz was tested in a two generation (two litters) study in SD rats. Administration was via the diet corresponding to mean achieved doses of approx 0, 3, 13 and 57 mg/kg/day for parents and 0, 4, 17 and 75 mg/kg/day for offspring. Parents were exposed for 9 weeks before mating, and representative offspring were retained to form a second generation. The initial animals were then re-mated with different males and females. Animals forming the second	Cozens *et al* (1982) (Reported in JMPR, 2001)

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
			generation were mated about 8 weeks after selection and were also re-mated with different male and female pairings. No. of animals on study not given but 10/sex/group were examined. There was evidence of systemic toxicity at the highest dose *e.g.* piloerection, pallor, decreased body weight. At this dose, there was evidence of dystocia in both generations but no effects on pregnancy rates. There were no obvious effects on male mating performance. Also at this dose, impaired growth of the offspring to weaning was noted. The incidence of structural anomalies recorded at terminal macroscopic examination of the remaining F1a and F2a pups and all F1b and F2b offspring provided no indication of any adverse effect of treatment. Increased mean liver weights occurred in all treated weanling and adult offspring. No histopathological changes in endocrine tissues were considered to be attributable to treatment. Sexual maturation was not determined. Reproductive performance was affected only at the highest dietary concentration, which was toxic to the parent animals. The NOAEL was 3 mg/kg/day. The study was considered to be negative for effects on reproduction and endocrine endpoints.	
C.4.7	Extended One-Generation Reproductive Toxicity Study (draft OECD TG 443)		No data available	
	B. Assays that have not yet completed validation, or have not primarily been designed for detection of			

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
	endocrine disruption, for which limited guidance is given in Annex 2 of the GD			
	***In vitro* Screens**			
Annex 2.1	The Human AR Transcriptional Activation Assay for Detection of Androgen (Ant)agonist-Activity of Chemicals (AR STTA)	+	Transient transfection assay (CHO cells) with luciferase as reporter gene. Prochloraz was tested in the presence and absence of R1881. No agonism detected but antagonism demonstrated with LOEC of 10 µM at which the response with R1881 was reduced from 100% to 36%.	Andersen *et al* (2002)
		+	System as above. IC50 was 3.5µM at which the response with R1881 was reduced from 100% to 35%.	Birkhoj *et al* (2004)
		+	Stably transfected MDA-KB2 cell line with luciferase as reporter gene. Prochloraz was tested for antagonism in the presence of various androgens. IC50 was 3.4-5.0 µM depending upon androgen agonist used.	Blake *et al* (2010)
		+	Stably transfected PALM cells. Prochloraz was tested for antagonism in 2 labs. IC50s of 3.8-7.8 µM obtained in one lab, antagonism demonstrated in 2nd lab but unclear dose-response prevented calculation of IC50.	Freyberger *et al* (2010b)
		+	Assay system as described in Andersen *et al* (2002). Prochoraz antagonised R1881 with LOEC of 6.3µM and IC50 of 13µM.	Kjaerstad *et al* (2010)
		+	Stably transfected MDA-KB2 cell line with luciferase as reporter gene. Prochloraz antagonistic in the presence of DHT. IC50 was between 3 & 10 µM.	Noriega *et al* (2005)
		+	Stably transfected CALUX cells. Prochloraz was tested for antagonism in 2 labs. IC50s of 1.46 and 2.06 µM were obtained.	Van der Burg *et al* (2010)

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
		+	System similar to Andersen *et al* (2002). Prochoraz antagonised R1881 with IC50 of 3.6µM. Metabolites 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol had no effect.	Vinggaard *et al* (2002)
	Wildlife Screens and Tests			
Annex 2.2	Fish (Medaka) Multi-Generation Test (MMGT)		ED effects only. Statistics by HISTOGEN (John Green et.al.). Treatment concentrations µg/L, 0±SD: 5±1, 9±2,17±3, 25±4, and 41±3. N.B. Histopathology data in review. Will be added soon **Sub-adults** (8 wk pf): **F1**	Unpublished USEPA data (2011)
		+	♂**Anal fin papillae** ↓ (LOEC = 9 µg/L; NOEC = 5 µg/L).	
		+	♀**VTG** ↓ (LOEC = 9 µg/L; NOEC = 5 µg/L). **F2**	
		+	♂**Anal fin papillae** (NOEC = 17 µg/L).	
		+	♀**VTG** ↓ (LOEC = 9 µg/L; NOEC = 5 µg/L). **Adults** (16 wk pf):	
		+	**F0 Fecundity** ↓ (LOEC = 41 µg/L; NOEC = 25 µg/L) **F1 Fecundity** (NOEC > 25 µg/L)	
		+	**F2 Fecundity** ↓ (LOEC = 25 µg/L; NOEC = 17 µg/L)	
Annex 2.3	Larval Amphibian Growth and Development Assay (LAGDA)		No data available (test in progress with USEPA)	
Annex 2.4	Avian Two Generation Test (ATGT)		No data available	
	Mammalian Screens and Tests			
Annex 2.5	Adult Male Assay		No data available	
Annex 2.6	TG 408: Repeated Dose 90 Day Oral Toxicity Study in Rodents		**Standard Assay:** No data available	
		+	**Non-Standard Assay:** Male and female CD-1 mice were given diets providing doses of prochloraz of 0,	Gale (1980) (Reported in JMPR, 2001)

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
			6, 25, 100 or 400 mg/kg/day for 6 (n=9) or 13 (n=15) weeks. A control group comprised 24 mice of each sex. Some signs of systemic toxicity occurred at 400 mg/kg/ day, *e.g.* piloerection and reduction in weight gain. At week 6, liver weight was increased in both sexes at 100 or 400 mg/kg/day and was slightly increased in females at 25 mg/kg/day. The ovaries of some females at the highest dose and in most at 100 mg/kg/day were small, and the prostate and seminal vesicles of males at 400 mg/kg/day were small. At week 13, the weight of the liver was increased in both sexes at 25, 100 or 400 mg/kg/day. The ovaries were small in females and the prostate and seminal vesicles small in males at 400 mg/kg/day. The NOAEL was 6 mg/kg/day based on effects in the liver at the next higher dose.	
		+	Male and female Wistar rats were given prochloraz by gavage at doses of 0, 6, 25 or 100 mg/kg/day for 6 (n=10) or13 (n=20) weeks. At week 6, liver weight was increased in both sexes at 100 mg/kg/day and in females given 6 or 25 mg/kg/day. The prostate and seminal vesicles were smaller than usual in males and the ovaries were larger in females at 100 mg/kg/day. The thyroid weight was increased in all treated females. At week 13, liver weight was increased in males at 6 mg/kg/day and in both sexes at 25 and 100 mg/kg/day; the ovary weight was increased in all treated females; and the thyroid weight was increased in females at 6 and 100 mg/kg/day. A NOAEL could not be established.	Shaw (1979) (Reported in JMPR, 2001)
Annex 2.7	OECD TG 453: Combined Chronic Toxicity/Carcinogenicity Studies	-	**Standard Assay:** Male and female mice (n=52) were fed diets containing prochloraz at concentrations of 0, 78, 325 or 1300	Colley *et al* (1983) (Reported

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
	(including TG 452 Chronic Toxicity Studies)		ppm, corresponding to doses of 0, 7.5, 33 and 130 mg/kg/day for males and 8.8, 36 and 150 mg/kg/day for females. Males were treated for 106 weeks and females for 121 weeks. A dose-related increase in liver tumours (adenomas and carcinomas) occurred in both sexes. No treatment-related effect was detected on the incidence of tumours at any other site or any other non-neoplastic changes recorded. The NOAEL was 78 ppm (7.5 mg/kg/day) based on liver tumours.	in JMPR, 2001)
		-	Male and female SD rats (n=60) were fed diets containing prochloraz at concentrations of 0, 38, 150 or 625 ppm, corresponding to doses of 1.3, 5.1 and 22 mg/kg/day for males and 1.6, 6.4 and 28 mg/kg/day for females. Males were treated for 115 weeks and females for 111 weeks. Separate groups of rats were killed after 52 weeks, and additional groups of controls and rats at the highest concentration, were killed after 13 weeks of treatment. After 13 weeks, increased liver weight was seen at the top dose and by 52 weeks, at 150 mg/kg/day too. There were no treatment-related increased incidences of tumours at any site. The NOAEL was 38 ppm (1.3 mg/kg/day), based on hepatic effects (periportal glycogen loss and centrilobular fat deposition) at the next highest dose. **Non-Standard Assay:** No data available	Colley *et al* (1983) Reported in JMPR, 2001)
Annex 2.8	OECD TG 421 Reproduction/Developmental Toxicity Screening Test and TG 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity		No data available	

GD Section	Tests and Screens	Result (+ - Eq)	Data for prochloraz	Reference
	Screening Test			

Table 4.1.2: Data for non-standardised assays used in the case study on prochloraz for GD No. 150.

In vitro and *in vivo* data are given separately (under the headings in the "Building Blocks" within the GD) and are listed in alphabetical order of author.

Summary of Prochloraz Data	**Reference**
Mechanism (*in vitro* mechanistic data)	
MCF-7 cell proliferation assay for estrogen agonists and antagonists. Prochloraz was tested in the presence and absence of estradiol. No agonism was detected but antagonism was evident with a LOEC of 1.0 μM and 47% inhibition of the maximum response with estradiol.	Andersen *et al* (2002).
Rat pituitary GH3 cell proliferation assay for TH agonists and antagonists. Prochloraz inhibited cell growth both alone and in the presence of T3 with a LOEC of 10 nM.	Ghisari and Bonefeld-Jorgensen (2005)
In the MCF7-BUS cell system of Hofmeister, and Bonefeld-Jorgensen (2004), co-exposure of prochloraz with E2 caused a significant increase in ERβ mRNA level.	Grunfeld and Bonefeld-Jorgensen (2004)
In MCF7-BUS cells, prochloraz down regulated the expression of ERα and ERβ mRNAs and proteins.	Hofmeister, and Bonefeld-Jorgensen (2004)
MCF-7 cell proliferation assay. Prochloraz was tested for ER antagonism by co-exposure with E2 and for aromatase inhibition by co-exposure with T (MCF7 cells express aromatase activity naturally and therefore T causes proliferation). Antagonism of E2 occurred (LOEC 3.1 μM) and antagonism of T (aromatase inhibition) (LOEC 1 μM).	Kjaerstad *et al* (2010)
Prochloraz inhibited 5alpha-reductase activity in two *in vitro* assays: an enzyme assay with human Lymph Node Carcinoma of Prostate (LNCaP) cells and an enzyme assay with human prostate tissue homogenate.	Lo *et al* (2007)
CALUX assay (rat and human cell based reporter gene assays) for AhR agonism and antagonism. Prochloraz was tested in the presence and absence of TCDD. No antagonism was detected but agonism was evident with a LOEC of 1.0 μM and a	Long *et al* (2003).

response of 140-531% of control values (dependent upon the cell type).	
In H295R cells, prochloraz caused a dose-dependent inhibition of cortisol secretion and a biphasic effect on aldosterone secretion, with a 2-fold stimulation at low concentrations and a strong inhibition at high concentrations.	Ohlsson *et al* (2009)
Prochloraz) showed AhR-mediated transcriptional activity in an AhR-mediated reporter cell line, DR-EcoScreen cells, (stably transfected mouse hepatoma Hepa1c1c7 cells).	Takeuchi *et al* (2008)
In H295R cells, prochloraz inhibited cortisol secretion, in the presence or absence of forskolin stimulation.	Ulleras *et al* (2008)
Fathead minnow (*Pimephales promelas*) ovary explants were incubated for 14.5 h with dilutions of prochloraz. Both E2 and T production were depressed, with IC50 values of 1.6 µM and 3.5 µM, respectively. A human adenocarcinoma H295R steroidogenesis assay also showed depressed E2 and T production at concentrations similar to those seen in ovary explants. These results suggest that, in fish, prochloraz inhibits one or more enzymes upstream of aromatase, as well as aromatase itself, although the latter appears to be more sensitive. The H295R assay appeared to be about 10 times more sensitive than effects on fecundity *in vivo* (Ankley *et al*. 2005), while the ovary explant assay was less sensitive.	Villeneuve *et al*. (2007)
MCF-7 cell proliferation assay for estrogen agonists. Prochloraz was not an agonist (antagonism not tested). Also tested in YES assay but was toxic.	Vinggaard *et al* (1999)
Effects (*in vivo* effects of concern) **Studies in non-mammalian species : Fish**	
Adult fathead minnow (*Pimephales promelas*) were exposed to prochloraz (30-300 µg/L) for 8 days followed by 8 d depuration, and various effects on HPG axis function were measured. In females, 30 µg/L caused transient depression of ex-vivo ovarian estradiol production, while 300 µg/L caused permanent E2 depression, as well as up to 3-fold VTG depression, while exposure lasted. In males, prochloraz caused depression of testosterone production at both concentrations. Both these effects disappeared soon after exposure ceased. Several genes associated with steroidogenesis were upregulated in both sexes.	Ankley *et al*. (2009)
Prepubertal male rainbow trout (*Oncorhynchus mykiss*) were exposed for 15 days to 7.9-66 µg/L prochloraz. This caused inhibition of spermatogenesis as shown by the stage of gonadal development reached 3 weeks after exposure. This effect was reversible within 9 weeks post-exposure.	Le Gac *et al*. (2001)
Adult female zebrafish were exposed to 300 µg/L prochloraz. Decreased plasma T and E2 concentrations and corticotrophin-releasing hormone (CRH) were seen after 12 and 48 h. The decrease in plasma E2 caused by prochloraz was correlated with the down-regulation of CRH mRNA expression.	Liu *et al* (2011)
Modelling work based on fathead minnow (*Pimephales promelas*) data *inter alia* from a study of prochoraz (Ankley *et al*., 2005) showed that VTG depression is correlated with reductions in fecundity. A reduction in plasma VTG as small as 25% was predicted to	Miller *et al*. (2007)

be associated with major reductions in fathead minnow population size after 1-5 years.	
Adult female fathead minnow (*Pimephales promelas*)were exposed to prochloraz at 300 µg/L for up to 24 h. Plasma E2 declined within 6 h and ex vivo E2 production was reduced at all time points, with no change in T. Plasma VTG was reduced by 2-fold at 24 h. Genes coding for CYP19, CYP17 and steroid acute regulatory (STAR) protein were up-regulated in ovary in a compensatory manner. Overall, the results are consistent with compensation of the HPG axis to inhibition of steroidogenesis by prochloraz.	Skolness *et al*. (2011)
Medaka (*Oryzias latipes*) were exposed to prochloraz (30 and 300 µg/L) for 7 days. Both 30 and 300 µg/L caused significant reductions in fecundity by up to 5-fold. This was associated with up-regulation of ovarian CYP17 and 19A genes, and down-regulation of various female hepatic genes including ERα, VTG I and II, and several choriogenin genes.	Zhang *et al*. (2008)
Studies in non-mammalian species : Amphibians	
Common frog (*Rana temporaria*) were exposed to prochloraz (11, 115 and 252 µg/L) from hatch to metamorphosis. The proportion of males was increased by approx. 5-fold (LOEC = 115 µg/L; NOEC = 11 µg/L) and the proportion of hermaphrodites decreased approx. 3-fold (LOEC = 115 µg/L; NOEC = 11 µg/L). Also, females were absent from all treatment groups. Whole-body testosterone levels were reduced by the two highest prochloraz treatments (LOEC = 115 µg/L; NOEC = 11 µg/L), suggesting that enzymes upstream of aromatase were being affected in addition to aromatase itself.	Brande-Lavridsen *et al*. (2008)
Studies in mammalian species	
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 = 865nM). 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). These 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	Blystone *et al* (2007)
Prochloraz was administered orally to pregnant rats in a mixture study but also including single substance experiments. Rats were dosed at 5, 25 & 50 mg/kg/day, from gestational day 7 to PND 16 and male offspring examined. Changes observed included changes in AGD, retained nipples and sex organ weights, with a NOAEL of 5 mg/kg/day.	Christiansen *et al* (2009)
Prochloraz was administered orally to pregnant rats at doses of 50 & 150 mg/kg/day, from gestational day 7 to PND 16. Male and female offspring were examined, a subset of foetuses were examined after Caesarian section of dams at gestational day 21. Prochloraz caused mild dysgenesis of the male external genitalia, reduced AGD and	Laier *et al* (2006)

retention of nipples in male pups. In female pups AGD was increased AGD. In male foetuses, testicular and plasma levels of T were decreased and levels of P increased. Immunohistochemistry of fetal testes showed increased expression of 17alpha-hydroxylase/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.	
Rats treated for 3 days with prochloraz (250 mg/kg/day) had increased hepatic total cytochrome P-450 and levels of associated drug metabolising enzymes (*e.g.* pentoxyresorufin O-depentylase) indicating that prochloraz is a "mixed inducer" of the hepatic cytochromes P-450. Prochloraz also showed tight binding to the haemoprotein of cytochrome P-450.	Laignelet *et al* (1989)
Single oral doses of [^{14}C]- prochloraz in the rat (50 and 250 mg/kg), resulted in almost 100% excretion in the urine or faeces within 96 hr of dosing. Urinary elimination accounted for about 65% of the dose and prochloraz was completely metabolised. The main biotransformation products in were 2,4,6-trichlorophenoxyacetic acid and its corresponding alcohol glucuronide. Other minor products were also identified.	Laignelet *et al* (1992)
Prochloraz administered orally to rats (100 mg/kg) was extensively metabolised. The major metabolites were 2,4,6-trichlorophenoxyacetic acid and 2-(2,4,6-trichlorophenoxy)ethanol, (present mainly as a glucuronide). Ring hydroxylation occurred to produce several minor metabolites. No unchanged prochloraz was excreted in the urine. Metabolites were excreted within 96 h, with greater than 50% excreted within 24h.	Needham *et al* (1991)
Prochloraz and three of its major metabolites induced the rat hepatic mixed-function oxidase system, giving mixed induction profiles. Prochloraz increased aldrin epoxidase and 7-pentoxyresorufin-O-dealkylase (7- and 14-fold respectively) and gave a slight increase in lauric acid hydroxylase. N-Propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]urea, a primary intermediate in the metabolism of prochloraz, also increased the activity of aldrin epoxidase and 7-pentoxyresorufin-O-dealkylase. Trichlorophenoxyethanol and trichlorophenoxyacetic acid both increased the activity of lauric acid 12-hydroxylase.	Needham *et al* (1992)
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, AGD adjusted\for body weight was not affected, but in females adjusted AGD was increased at 250 mg/kg. In females VO was unaffected. Nipple retention 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 SATs 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.	Noriega *et al* (2005)
Prochloraz was administered orally to pregnant rats at a dose of 30 mg/kg/day, from	Vinggaard *et al*

gestational day 7 to PND 16. Male and female offspring were examined, a subset of foetuses were examined after Caesarian 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. Overall these results strongly indicate that prochloraz feminizes the male offspring after perinatal exposure, and that these effects are due, at least in part, to diminished fetal steroidogenesis.	(2005b)

1.1. Analysis of Building Blocks Using Prochloraz Data

17. Each assay for which guidance is given in the draft GD has been evaluated using the relevant data for prochloraz inserted into the building block in the draft GD. The approach used is described in Section 3.2 (above). The wording in each series of tables is designed to mirror that used in the building blocks (BBs) in the draft GD. The results of the assays have been abbreviated for here clarity but more detail is given in Tables 4.1.1 and 4.1.2.

1.2. Prochloraz Case Study Results: Validated *in vitro* Assays

1.2.1. ER Binding Assay (US EPA OPPTS 890.1250) (GD No. 150 Section C.2.2)

There are no data available

1.2.2. AR Binding Assay (US EPA OPPTS 890.1150) (GD No. 150 Section C.2.1)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows binding to AR, while existing *in vitro* data show a variety of responses including ER and AR antagonism, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.3.2.1: Prochloraz data summary

AR Binding assay data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Prochloraz displaced R1881 with IC50 of approximately 60µM. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as	

AR Binding assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		reported in JMPR, 2001).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	Pre/postnatal dosing (250 -50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.3.2.2: Conclusions about AR Binding Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Prochloraz interacts with AR combined with effects on ER, S (and possibly T). Potential for adverse effects via multiple mechanisms	Perform assay AR STTA (level 2)	AR STTA: positive Antagonism, IC50 3-10 µM. (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).
	or assay from upper levels *e.g.* H assay (level 3)	H assay : positive AR antagonism. Reductions in SAT weights at 50 mg/kg/day and above (no effects at 25 mg/kg/day). (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004).
	or fish screen (AFSS) (level 3)	Not available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
	or male PP assay (level 4)	Male PP assay : positive AR antagonism & S disruption. PPS was delayed and SAT weights were reduced at 62 mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).
	or ext-1 or 2-gen assays (level 5)	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).
	or partial/full fish life cycle tests (level 4/5).	FSDT and MMGT: both positive ♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓ (suggest AR and ER antagonism & S disruption). (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data)

Table 4.3.2.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz

Other considerations (as given in BB)	Conclusions for Prochloraz
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) study on prochloraz is inadequate for assessment (Cozens *et al*, 1982) but literature pre/post natal studies provide sufficient information to conclude evidence of concern for ED (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b). If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more

Other considerations (as given in BB)	Conclusions for Prochloraz
	endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention).
If existing data are from level 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g*. FSDT) may be sufficient for this purpose.	Male PP assay alone (level 4) on prochloraz would not provide sufficient data for ED assessment (Blystone *et al*, 2007) but would when combined with adequate level 5 (or literature pre & post natal studies) (Blystone *et al*, 2007 *etc*).
If existing data are from H assay or AFSS then level 4 mammalian assay or fish screen (TG 229/230) will provide data on multiple modalities.	H assay on prochloraz demonstrates AR antagonism *in vivo* (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004). FSDT and MMGT suggest AR and ER antagonism & S disruption (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data)
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	The prochloraz metabolites 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol had no effect in the AR STTA assay whilst prochloraz was an antagonist (Vinggaard *et al*, 2002). Indicates detoxification by metabolism.

18. Overall conclusions about AR Binding Assay and existing data: Next steps and other considerations provide a logical course of action to follow. 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 H assay provides confirmation that this mechanism may act in vivo, whilst the positive results in the level 4 male PP assay and fish assays suggest that A, S or E modailities may be responsible for the effects seen on endocrine endpoints. The combined dataset provides sufficient evidence of endocrine activity.

4.3.3. OECD TG 455: The Stably Transfected Human ERα Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA) (including Guidance for the Antagonism Assay) (GD No. 150 Section C.2.3)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows antagonism of ER in reporter gene assays,

while existing *in vitro* data show a variety of responses including AR antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.3.3.1: Prochloraz data summary

Human ERα Transcriptional Activation assay data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Prochloraz antagonised activation of the reporter gene with E2. LOEC was approx 10µM. No agonism detected. Assay system non-standard (predates OECD TG 455). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005).	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	ER antagonism but no agonism (MCF7 cell proliferation assay). (Andersen *et al*, 2002; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006;	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats	

Human ERα Transcriptional Activation assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	OECD, 2010; Villeneuve *et al*, 2007).	(highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)		
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.3.3.2: Conclusions about Human ERα Transcriptional Activation assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Prochloraz antagonizes ER combined with effects on AR, S (and possibly T). Potential for	Perform assay from upper levels *e.g.* UT assay (level 3)	None

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
adverse effects via multiple mechanisms.		
	or female PP assay (level 4)	None
	or ext-1 or 2-gen assays (level 5)	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).
	or partial/full fish life cycle tests (level 4/5).	FSDT and MMGT: both + ♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓ (suggest AR and ER antagonism & S disruption) (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data)

Table 4.3.3.3: Other considerations and conclusions for prochloraz

Other considerations (as given in BB)	conclusions for Prochloraz
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) study on prochloraz is inadequate for assessment (Cozens *et al*, 1982) but literature pre/post natal studies provide sufficient information to conclude evidence of concern for ED (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b). If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention).
If existing data are from level 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g*. FSDT) may be sufficient for this purpose.	Male PP assay alone (level 4) on prochloraz would not provide sufficient data for ED assessment (Blystone *et al*, 2007) but would when combined with adequate level 5 (or literature pre & post natal studies) (Blystone *et al*, 2007 *etc*).

Other considerations (as given in BB)	**conclusions for Prochloraz**
If existing data are from UT assay then level 4 mammalian assay or fish screen (TG 229/230) will provide data on multiple modalities.	No UT data available therefore no information about ER antagonism *in vivo*. H assay on prochloraz demonstrates AR antagonism *in vivo* (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004). As level 4 data and pre/post natal studies give adequate information for hazard assessment then further level 3/4 *in vivo* testing is not justified. FSDT and MMGT suggest AR and ER antagonism & S disruption (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data) .
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989,1992; Needham *et al* 1991, 1992).
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	The prochloraz metabolites 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol had no effect in the AR STTA assay whilst prochloraz was an antagonist (Vinggaard *et al*, 2002). Indicates detoxification by metabolism.

19. Overall conclusions about human ER STTA assay and existing data: Next steps and other considerations provide a logical course of action to follow. The combined dataset indicates that the ER and AR antagonism and S disruption shown *in vitro* also occur *in vivo* in mammals and fish. Although the ER STTA assay indicates that prochloraz is an ER antagonist, a UT assay was not available so ER antagonism was not tested in this *in vivo* screen. However, the positive results in the level 4 fish assays suggest that A, S or E modalities may be responsible for the effects seen on endocrine endpoints.It is not ethically justified to test this *in vivo* in mammals in a UT assay or in further higher tier wildlife assays as the existing CF and literature studies provide sufficient evidence of endocrine activity.

4.3.4 OECD TG 456: H295R Steroidogenesis Assay (GD No. 150 Section C.2.4)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows steroidogenesis disruption, while existing *in vitro* data show a variety of responses including ER antagonism, AR binding and antagonism, aromatase inhibition, and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.3.4.1: Prochloraz data summary

H295R Steroidogenesis assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused	

H295R Steroidogenesis assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).	nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	5α-reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.3.4.2: Conclusions about H295R Steroidogenesis Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Prochloraz inhibits steroidogenesis, in combination with effects on ER, AR, S (and possibly T). Potential for adverse effects via multiple mechanisms	Perform assay from upper levels *e.g.* male or female pubertal assay (level 4)	Male PP assay : positive AR antagonism & S disruption. PPS was delayed and SAT weights were reduced at 62 mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).
	or ext-1 or 2-gen assays (level 5)	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).
	or partial/full fish life cycle tests (level 4/5).	FSDT and MMGT: both + ♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓ (suggest AR and ER antagonism & S disruption) (Kinnberg *et al*.,

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
		2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data)

Table 4.3.4.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	conclusions for Prochloraz
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) study on prochloraz is inadequate for assessment (Cozens *et al*, 1982) but literature pre/post natal studies provide sufficient information to conclude evidence of concern for ED (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b). If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention).
If existing data are from level 3 or 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g*. FSDT) may be sufficient for this purpose.	Male PP assay alone (level 4) on prochloraz would not provide sufficient data for ED assessment (Blystone *et al*, 2007) but would when combined with adequate level 5 (or literature pre & post natal studies). Level 3 mammalian studies (H and UT assays) do not detect steroidogenesis disruption. FSDT and MMGT suggest AR and ER antagonism & S disruption (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data) .
Compare steroidogenesis assay results with other *in vitro* results to help discern mechanism.	Prochloraz is also positive for aromatase inhibition (USEPA, 2007 *etc*). This is consistent with the results of the H295R assay.
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive	The prochloraz metabolites 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol had no effect in the AR STTA

Other considerations (as given in BB)	conclusions for Prochloraz
results *in vitro* and *in vivo*.	assay whilst prochloraz was an antagonist (Vinggaard *et al*, 2002). Indicates detoxification by metabolism.

20. Overall conclusions about H295R Steroidogenesis Assay and existing data: Next steps and other considerations provide a logical course of action to follow. 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 positive results in the level 4 male PP assay 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.

4.3.5. Aromatase Assay (US EPA OPPTS 890.1200) (GD No. 150 Section C.2.5)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows aromatase inhibition while existing *in vitro* data show a variety of responses including ER antagonism, AR binding and antagonism, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.3.5.1: Prochloraz data summary

Aromatase assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
Prochloraz inhibited aromatase activity, using a number of enzyme sources. IC50s for human placental and human recombinant enzyme were 0.0284 and 0.0285 µM respectively). (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et*	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979;	

Aromatase assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
al, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).		as reported in JMPR, 2001).	
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.3.5.2: Conclusions about Aromatase Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Prochloraz inhibits aromatase, in combination with effects on ER, AR S (and possibly T). Potential for adverse effects via multiple mechanisms	Perform assay from upper levels *e.g.* male or female pubertal assay (level 4)	Male PP assay : positive AR antagonism & S disruption. PPS was delayed and SAT weights were reduced at 62 mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).
	or ext-1 or 2-gen assays (level 5)	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).
	or partial/full fish life cycle tests (level 4/5).	FSDT and MMGT: both + ♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓ (suggest AR and ER antagonism & S disruption) (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data)

Table 4.3.5.3: Other considerations and conclusions for prochloraz about Aromatase Assay and existing data; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	conclusions for Prochloraz
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) study on prochloraz is inadequate for assessment (Cozens *et al*, 1982) but literature pre/post natal studies provide sufficient information to conclude evidence of concern for ED (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005;

Other considerations (as given in BB)	conclusions for Prochloraz
	Vinggaard *et al*, 2005b). If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention).
If existing data are from level 3 or 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g*. FSDT) may be sufficient for this purpose.	Male PP assay alone (level 4) on prochloraz would not provide sufficient data for ED assessment (Blystone *et al*, 2007) but would when combined with adequate level 5 (or literature pre & post natal studies). Level 3 mammalian studies (H and UT assays) do not detect steroidogenesis disruption. FSDT and MMGT suggest AR and ER antagonism & S disruption (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data) .
Compare aromatase assay results with other *in vitro* results to help discern mechanism.	Prochloraz is also positive in the H295R assay for steroidogenesis disruption (OECD, 2010 *etc*). This is consistent with the results of the aromatase assay.
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	The prochloraz metabolites 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol had no effect in the AR STTA assay whilst prochloraz was an antagonist (Vinggaard *et al*, 2002). Indicates detoxification by metabolism.

21. Overall conclusions about Aromatase Assay and existing data: Next steps and other considerations provide a logical course of action to follow. 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 positive results in the level 4 male PP assay 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.

1.3. Prochloraz Case Study Results: Validated Wildlife *in vivo* Assays

1.3.1. OECD TG 229: Fish Short Term Reproduction Assay (FSTRA) (GD No. 150 Section C.3.1)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +
Scenario A is appropriate because the assay shows depressed ♀ VTG as well as effects on secondary sexual characteristics and fecundity, while existing *in vitro* data show a variety of responses including aromatase inhibition and steroidogenesis disruption, and existing *in vivo* data show a variety of endpoints consistent with interference with steroidogenesis.

Table 4.4.1.1: Prochloraz data summary

Fish Short Term Reproduction Assay data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
♀ VTG ↓ (3-fold); ♂ secondary sexual characteristics ↓; fecundity ↓ (3 to 5-fold) NOECs for effects on VTG = <20-30 µg/L, on secondary sexual characteristics = 34 µg/L, and on fecundity = 16-100 µg/L. (Ankley *et al*. 2005; Biever *et al*., 2007; Jensen & Ankley, 2006)	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	TG 230: ♀VTG↓ in 2 fish species (NOEC = 38-58 µg/L) (OECD, 2006)
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	Non-standard short-term studies with several fish species show effects on various endocrine-related endpoints at concentrations ranging from 8 to 300 µg/L. These are consistent with the results of TG 229. (Ankley *et al*., 2009; Le Gac *et al*., 2001; Liu *et al*., 2011; Skolness *et al*., 2011; Zhang *et al*., 2008)
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	A non-standard short-term study with frog larvae revealed an increased proportion of males at 115 µg/L. These are also consistent with the results of TG 229. (Brande-Lavridsen *et al*. 2008)

Fish Short Term Reproduction Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)		
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.4.1.2: Conclusions about Fish Short Term Reproduction Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Strong evidence for *in vivo*	Consider performing a fish	MMGT: +

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
endocrine activity with potential adverse effects (reproductive toxicity) in fish	lifecycle test, especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx. [In this case, a decision to perform fish lifecycle testing is sensible given the weight of *in vitro* and *in vivo* data suggesting the occurrence of endocrine disruption in mammals, fish and amphibians.]	♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓ (suggest AR and ER antagonism & S disruption). The MMGT NOEC for adverse effects (on fecundity of F0 and F2 fish) is 25 and 17 µg/L, respectively. Note that the FSTRA NOEC for effects on fecundity ranges from 16 to 100 µg/L.

Table 4.4.1.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	Conclusions for Prochloraz
An alternative approach would be to deploy the fish sexual development test, especially if sexual development is expected to give a response at lower concentrations than reproduction.	The FSDT NOEC for adverse effects (on sex ratio) ranges from 44 to 101 µg/L . In this case, the sex ratio endpoint appears to be less sensitive than the fecundity endpoint in the MMGT, but it clearly confirms that prochloraz causes adverse effects in fish at low concentrations.
The decision about whether to conduct a Fish Lifecycle Toxicity Test (FLCTT) or multi-generation test (MMGT) may be driven primarily by the bioaccumulative properties of the chemical – a one generation test (*e.g.* F0 eggs to F1 fry) will generally be sufficient if the chemical is not expected to be transferred to the young via the eggs.	Prochloraz is not strongly bioaccumulative, so a 1-generation FLCTT would have been an option (NB: FLCTT data are not available). Comparisons of the F0/F1 with the F2 MMGT data do not suggest that the F2 generation is substantially more sensitive than the F0/F1 generations to prochloraz, which provides support for taking the FLCTT option in this case.

22. Overall conclusions about Fish Short Term Reproduction Assay and existing data: Next steps and other considerations provide a logical course of action to follow, although the sensitivity of TG 229 to prochloraz is almost as high as a lifecycle test. In this case, the FSDT as a next step is somewhat less sensitive than full or multiple lifecycle testing. There appears to be scope for read-across from fish assays to amphibians.

4.4.2. OECD TG 230: 21 Day Fish Assay (GD No. 150 Section C.3.2)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows depressed ♀ VTG, while existing *in vitro* data show a variety of responses including aromatase inhibition and steroidogenesis disruption, and existing *in vivo* data show a variety of endpoints consistent with interference with steroidogenesis.

Table 4.4.2.1: Prochloraz data summary

21 Day Fish Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
♀ VTG ↓ (up to 10-fold) NOECs for effects on VTG = 38-58 µg/L (OECD, 2006)	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	TG 229: NOECs for effects on VTG = <20-30 µg/L, on secondary sexual characteristics = 34 µg/L, and on fecundity = 16-100 µg/L. (Ankley *et al*. 2005; Biever *et al*., 2007; Jensen & Ankley, 2006)
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	Non-standard short-term studies with several fish species show effects on various endocrine-related endpoints at concentrations ranging from 8 to 300 µg/L. These are consistent with the results of TG 230. (Ankley *et al*., 2009; Le Gac *et al*., 2001; Liu *et al*., 2011; Skolness *et al*., 2011; Zhang *et al*., 2008)
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	A non-standard short-term study with frog larvae revealed an increased proportion of males at 115 µg/L. These are also consistent with the results of TG 230. Brande-Lavridsen *et al*. 2008
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad	TG 453- no ED effects were noted in mice (highest dose was 150	

21 Day Fish Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	et al, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)		
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.4.2.2: Conclusions about 21 Day Fish Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Strong evidence for *in vivo* endocrine activity in fish and other organisms.	Consider performing a fish lifecycle test, especially if the intention is to obtain precise data	MMGT: + ♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
	on a reproductive or developmental NOEC/ECx. [In this case, a decision to perform fish lifecycle testing is sensible given the weight of *in vitro* and *in vivo* data suggesting the occurrence of endocrine disruption in mammals, fish and amphibians.]	(suggest AR and ER antagonism & S disruption). The MMGT NOEC for adverse effects (on fecundity of F0 and F2 fish) is 25 and 17 µg/L, respectively.

Table 4.4.2.3: Other considerations and conclusions for prochloraz. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	Conclusions for Prochloraz
An alternative approach would be to deploy the fish sexual development test, especially if sexual development is expected to give a response at lower concentrations than reproduction.	The FSDT NOEC for adverse effects (on sex ratio) ranges from 44 to 101 µg/L. In this case, the sex ratio endpoint appears to be less sensitive than the fecundity endpoint in the MMGT, but it clearly confirms that prochloraz causes adverse effects in fish at low concentrations.
The decision about whether to conduct a Fish Lifecycle Toxicity Test (FLCTT) or multi-generation test (MMGT) may be driven primarily by the bioaccumulative properties of the chemical – a one generation test (*e.g.* F0 eggs to F1 fry) will generally be sufficient if the chemical is not expected to be transferred to the young via the eggs.	Prochloraz is not strongly bioaccumulative, so a 1-generation FLCTT would have been an option (NB: FLCTT data are not available). Comparisons of the F0/F1 with the F2 MMGT data do not suggest that the F2 generation is substantially more sensitive than the F0/F1 generations to prochloraz, which provides support for taking the FLCTT option in this case.

23. Overall conclusions about 21 Day Fish Assay and existing data: Next steps and other considerations provide a logical course of action to follow. In this case, the FSDT as a next step is somewhat less sensitive than full or multiple lifecycle testing. There appears to be scope for read-across from fish assays to amphibians.

4.4.3. Variant of OECD TG 230: Androgenised Female Stickleback Screen (AFSS) (GD No. 150 Section C.3.3)

There are no data available.

4.4.4. Fish sexual development test (FSDT) (TG 234) (GD No. 150 Section C.3.4)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows depressed ♀ VTG and ♂–biased sex ratio, while existing *in vitro* data show a variety of responses including aromatase inhibition and steroidogenesis disruption, and existing *in vivo* data show a variety of endpoints consistent with interference with steroidogenesis.

Table 4.4.4.1: Prochloraz data summary

Fish Sexual Development Test data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
♀ VTG ↓ 4 to 1000-fold (NOEC = 15-64 µg/L) Proportion of ♂ and intersexes ↑ (NOEC = 44-64 µg/L) Proportion ♀ ↓ 2 to 8-fold (NOEC = 44-101 µg/L. (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; Holbech *et al*., 2011)	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	TG 229: NOECs for effects on VTG = <20-30 µg/L, on secondary sexual characteristics = 34 µg/L, and on fecundity = 16-100 µg/L. (Ankley *et al*. 2005; Biever *et al*., 2007; Jensen & Ankley, 2006)
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	TG 230: ♀VTG↓ in 2 fish species (NOEC = 38-58 µg/L) (OECD, 2006)
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	Non-standard short-term studies with several fish species show effects on various endocrine-related endpoints at concentrations ranging from 8 to 300 µg/L. These are consistent with the results of TG 230. (Ankley *et al*., 2009; Le Gac *et al*., 2001; Liu *et al*., 2011; Skolness *et al*., 2011; Zhang *et al*., 2008)

Fish Sexual Development Test data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	A non-standard short-term study with frog larvae revealed an increased proportion of males at 115 µg/L. These are also consistent with the results of TG 230. (Brande-Lavridsen *et al*. 2008)
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	TG 441 (H assay) - AR antagonism. Reductions in SAT weights at 50 mg/kg/day and above (no effects at 25 mg/kg/day). (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004).	
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-reductase inhibition Lo *et al*, 2007).	Male pubertal assay - PPS was delayed and SAT weights were reduced at 62 mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day.	
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Fish Sexual Development Test data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		(Blystone *et al*, 2007).	

Table 4.4.4.2: Conclusions about Fish Sexual Development Test and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Strong evidence for adverse effects in fish and other organisms by an endocrine mechanism.	Some regulatory authorities may consider that further evidence is not required, especially as adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish lifecycle test should be considered. [In this case, a decision to perform fish lifecycle testing is sensible given the weight of *in vitro* and *in vivo* data suggesting the occurrence of endocrine disruption in mammals, fish and amphibians.]	MMGT: + ♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓ (suggest AR and ER antagonism & S disruption). The MMGT NOEC for adverse effects (on fecundity of F0 and F2 fish) is 25 and 17 µg/L, respectively.

Table 4.4.4.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	Conclusions for Prochloraz
If the FSDT was only performed with 3 test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx.	In several cases, the FSDT was run with 5 concentrations of prochloraz, so those NOECs could be considered reliable.
Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case the FSDT would be less responsive than a lifecycle test.	The FSDT NOEC for adverse effects (on sex ratio) ranges from 44 to 101 µg prochloraz /L . In this case, the sex ratio endpoint appears to be less sensitive than the fecundity endpoint in the MMGT, but it clearly confirms that prochloraz causes adverse effects in fish at low concentrations.
A decision about whether to conduct FLCTT or MMLC may be driven primarily by the bioaccumulative properties of the chemical – a one	Prochloraz is not strongly bioaccumulative, so a 1-generation FLCTT would have been an option (NB: FLCTT data are not available). Comparisons

Other considerations (as given in BB)	Conclusions for Prochloraz
generation test (*e.g.* F0 eggs to F1 fry) will generally be sufficient if the chemical is not expected to be transferred to the fry via the eggs.	of the F0/F1 with the F2 MMGT data do not suggest that the F2 generation is substantially more sensitive than the F0/F1 generations to prochloraz, which provides support for taking the FLCTT option in this case.

24. Overall conclusions about Fish Sexual Development Test and existing data: Next steps and other considerations provide a logical course of action to follow. There appears to be scope for read-across from fish assays to amphibians, but note that no long-term amphibian data are available.

4.4.5. Fish Lifecycle Toxicity Test (FLCTT) (USEPA OPPTS 850.1500) (GD No. 150 Section C.3.5)

There are no data available.

4.4.6. OECD TG 231: Amphibian Metamorphosis Assay (AMA) (GD No. 150 Section C.3.6)

There are no data available.

4.4.7. OECD TG 206: Avian Reproduction Test (GD No. 150 Section C.3.7)

There are no data available.

1.4. Prochloraz Case Study Results: Validated Mammalian *in vivo* Assays

4.5.1. OECD TG 440: Uterotrophic Bioassay in Rodents (UT assay) (Including GD on the Use of the Assay to Screen for Anti-Estrogenicity) (GD No. 150 Section C.4.1)

There are no data available

4.5.2. OECD TG 441: Hershberger Bioassay in Rats (H Assay) (Including OECD GD for Weanling Hershberger Bioassay) (GD No. 150 Section C.4.2)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that prochloraz was positive for AR antagonism in H assays, while existing *in vitro* data show a variety of responses including AR and ER antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.5.2.1: Prochloraz data summary

Hershberger Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
Prochloraz was positive in the H castrated rat assay for antagonism. Reductions in SAT weights occurred at 50 mg/kg/day (no effects at 25 mg/kg/day). (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004).	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	

Hershberger Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)		
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-Reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.5.2.2: Conclusions about Hershberger Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Strong evidence that prochloraz has anti-A activity with (potential for) adverse effects via AR mechanism. Significant inhibition of 5-alpha reductase activity is not obvious in the H assay, as similar effects were seen on all SATs and the VP did not appear to be more sensitive.	Perform assay from upper levels *e.g.* male pubertal assay (level 4)	Male PP assay : positive AR antagonism & S disruption. PPS was delayed and SAT weights were reduced at 62 mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).
	or ext-1 or 2-gen assay (level 5).	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).

Table 4.5.2.3: Other considerations and conclusions for prochloraz. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	conclusions for Prochloraz
Check pattern of change across sex tissues for possible 5-α reductase inhibition.	Significant inhibition of 5-alpha reductase activity is not obvious as similar effects were seen on all SATs and the VP did not appear to be more sensitive (Vinggaard *et al*, 2005b). In the study of Blystone *et al* (2007) VP was not significantly affected.
If existing data are from level 4 or 5 (or less sensitive assays) then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Male PP assay alone (level 4) on prochloraz would not provide sufficient data for assessment of endocrine disruption (Blystone *et al*, 2007) but would when combined with adequate level 5 (or literature pre & post natal studies) (Blystone *et al*, 2007 *etc*). "Less sensitive assays" refers to to effects seen in repeat dose toxicity studies. Prochloraz reduced SAT weights in TG 407 & 408 assays. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive

Other considerations (as given in BB)	conclusions for Prochloraz
	evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention).
Consider route of exposures for H assay and existing effects data and possible implications of ADME characteristics of the chemical.	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	The prochloraz metabolites 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol had no effect in the AR STTA assay whilst prochloraz was an antagonist (Vinggaard *et al*, 2002). Indicates detoxification by metabolism.
A positive result could have arisen from other (EATS or non-EATS) mechanisms.	Prochloraz also has other effects, *e.g.* it affects cortisol (inhibition) and aldosterone (stimulation at low concs and inhibition at high concs) secretion *in vitro* (H295R cells) (Ohlsson *et al*, 2009; Ulleras *et al*, 2008). The positive result in the H assay is however, unlikely to have arisen via other mechanisms.

25. Overall conclusions about Hershberger Assay and existing data: next steps and other considerations provide a logical course of action to follow. H assay appeared to have similar sensitivity to male PP assay for SAT weights but serum T reduction gave a lower NOAEL in the male PP assay. The PP assay is also responsive to both A and S modulation. 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 other wildlife species although the physiological consequences of the effects are likely to be different.

4.5.3. Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats (Male PP Assay) (US EPA OPPTS 890.1500) (GD No. 150 Section C.4.3)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that prochloraz produced positive results in the male rat PP assay that were indicative of AR antagonism and/or S disruption. Existing *in vitro* data show a variety of responses including AR and ER antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.5.3.1: Prochloraz data summary

Male Rat PP Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
Prochloraz was positive in the male pubertal rat assay. Prochloraz had significant effects on both indicators of hormonal activity and apical endpoints. Age at PPS was increased and SAT weights were reduced. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	

Male Rat PP Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	TG 441 (H assay) - AR antagonism. Reductions in SAT weights at 50 mg/kg/day and above (no effects at 25 mg/kg/day). (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004).	
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-Reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.5.3.2: Conclusions about Male Rat PP Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay Result for Prochloraz
Increased evidence that prochloraz has (anti)-A activity and S disruption (weak, moderate or strong).	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).

Table 4.5.3.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other Considerations (as given in BB)	Conclusions for Prochloraz
If existing data are from level 5 then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) assay on prochloraz is not to current standards (Cozens *et al*, 1982) therefore would not provide sufficient data for assessment of endocrine disruption by itself. When combined with high quality literature pre & post natal studies (Blystone *et al*, 2007 *etc*) there is sufficient evidence of concern for endocrine disruption. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention).
Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints.	Consideration is valid but prochloraz affects both indicators of hormonal activity and apical endpoints.
Effects on apical endpoints alone may indicate EATS modalities or other mechanisms.	Consideration is valid but prochloraz affects both indicators of hormonal activity and apical endpoints.
Possible effects on E modality should also be considered.	Consideration is valid. Prochloraz has anti-E activity *in vitro* (*e.g.* Andersen *et al*, 2002) but effects via this mechanism cannot be dissociated from other effects in this apical assay.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).

26. Overall conclusions about Male Rat PP Assay and existing data: next steps and other considerations provide a logical course of action to follow. The male PP assay appeared to have similar sensitivity to the H assay for SAT weights but serum T reduction gave a lower NOAEL in the male PP assay. The PP assay is also responsive to both A and S modulation. 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.

4.5.4. Pubertal Development and Thyroid Function Assay in Peripubertal Female Rats (Female PP Assay) (US EPA OPPTS 890.1450) (GD No. 150 Section C.4.4)

There are no data available

4.5.5. OECD TG 407: Repeated Dose 28 Day Oral Toxicity Study in Rodents (GD No. 150 Section C.4.5)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that prochloraz produced positive results on endocrine endpoints in the male rat in a TG 407 assay, the data are indicative of AR antagonism. Existing *in vitro* data show a variety of responses including AR and ER antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.5.5.1: Prochloraz data summary

28 Day Repeat Dose Rodent Assay data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Although this is an old study (reported in 1980) prochloraz appears to have had positive results on apical endpoints. Indicators of hormonal activity were not determined. Prostate and seminal vesicles were described as "small" after prochloraz was administered orally at 100 and 400 mg/kg/day. No adverse histopathology was reported. (Lancaster & Shaw, 1980; as reported in JMPR,	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	

28 Day Repeat Dose Rodent Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
2001).	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).		
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)		
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-Reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.5.5.2: Conclusions about 28 Day Repeat Dose Rodent Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay Result for Prochloraz
Increased evidence that prochloraz has (anti)-A activity and S disruption (weak, moderate or strong).	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative.

Conclusions for Prochloraz	Next Step (as BB)	Assay Result for Prochloraz
		Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).

Table 4.5.5.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	Conclusions for Prochloraz
If existing data are from level 5 then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) assay on prochloraz is not to current standards (Cozens *et al*, 1982) therefore would not provide sufficient data for assessment of endocrine disruption by itself. When combined with high quality literature pre & post natal studies (Blystone *et al*, 2007 *etc*) there is sufficient evidence of concern for endocrine disruption. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention)..
Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints.	Consideration is valid but indicators of hormonal activity were not determined.
Effects on apical endpoints alone may indicate EATS modalities or other mechanisms.	Consideration is valid but only apical endpoints were determined.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).

27. Overall conclusions about 28 Day Repeat Dose Rodent Assay and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset indicates that the ER and AR antagonism and S disruption shown *in vitro* also occur *in vivo* in mammalian species. The TG 407, as an apical assay, has only a limited ability to discern mechanism but the positive result for antagonism in the H assay indicates that the effects on SATs may be a consequence of this mode of action. These effects also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different.

4.5.6. OECD TG 416: Two-Generation Reproduction Toxicity Study (GD No. 150 Section C.4.6)

Scenario J : Result -, existing *in vitro* data +, existing *in vivo* data +
Scenario J is appropriate because the assay indicates that prochloraz had no clear effects on endocrine endpoints in a TG 416 assay (old version of the TG). Existing *in vitro* data show a variety of responses including AR and ER antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.5.6.1: Prochloraz data summary

2-Generation Rat Reproduction Assay data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Study was not to current standards. Prochloraz had no obvious endocrine or reproductive effects at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. There were no effects on histopathology of reproductive organs. NOAEL was approx 3 mg/kg/day. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in	

2-Generation Rat Reproduction Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	(Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	TG 441 (H assay) - AR antagonism. Reductions in SAT weights at 50 mg/kg/day and above (no effects at 25 mg/kg/day). (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	Male pubertal assay - PPS was delayed and SAT weights were reduced at 62 mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).	
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-Reductase inhibition (Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.5.6.2: Conclusions about 2 Generation Rat Reproduction Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay Result for Prochloraz
Prochloraz treatment produced no evidence of adverse effects on reproduction/development/endocrine organs in TG 416. This study was conducted before the guideline was updated and therefore many endocrine sensitive endpoints were not determined. The multitude of effects seen in level 3 and 4 studies (H and PP assays) and detailed pre and postnatal mechanistic studies indicate that there is evidence of concern for endocrine disruption with prochloraz. Doseage differences may explain some of the differences from existing *in vitro* and *in vivo* data. Severe malformations were only observed in pre and post- natal studies where doses were above those used in the TG 416 study (Noriega *et al*, 2005). Other effects such as hormonal changes, nipple rete ntion, altered AGD and gene expression changes were detected in pre and post natal studies within the dose range used in the TG 416 study (Blystone *et al*, 2007; Christiansen *et al*, 2009, Laier *et al*, Vinggaard *et al*) but these are endocrine disruption sensitive endpoints that were not included in the TG 416 study.	If test is to current TG 416 standards, no further testing needed. If not then consider supplemental testing, depending upon existing data. To further discern mechanism could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	TG 416 (old version) not to current standards but the information available from PP assays (level 4) and detailed mechanistic literature studies (Blystone *et al*, 2007; Christiansen *et al*, 2009, Laier *et al*, Vinggaard *et al*) provides sufficient evidence that prochloraz poses concern for endocrine disruption. The data from these studies may be sufficient to derive a NOAEL for development (5 mg/kg/day). A NOAEL for reproduction could be provided by the existing TG 416 (3 mg/kg/day) as the test should have been adequate to detect effects on reproduction. Therefore there may be no need for further *in vivo* testing.

Table 4.5.6.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	Conclusions for Prochloraz
If existing data are from adequate *in vivo* studies such as 28d, 90d, chronic/carcinogenicity studies, than question why differences.	Age of TG 416 study (Cozens *et al*, 1982) and lack of sensitive endpoints for endocrine disruption explains differences.
Note that the ext-1 gen assay provides the most information on endocrine disruption.	An ext-1-gen assay (TG 443) would provide the most extensive evaluation of the endocrine disruption potential of prochloraz.
Consider route of exposures and possible implications for ADME characteristics of the chemical with existing studies.	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively

Other considerations (as given in BB)	Conclusions for Prochloraz
	metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).
Further mechanistic studies with metabolism may help determine MoA.	Not necessary as adequate mechanistic data exist.

28. Overall conclusions about 2 Generation Rat Reproduction Assay and existing data: next steps and other considerations provide a logical course of action to follow. 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.

4.5.7. Extended One-Generation Reproductive Toxicity Study (draft OECD TG 443) (GD No. 150 Section C.4.7)

There are no data available

1.5. Prochloraz Case Study Results: *In vitro* Assays that Have Not Yet Completed Validation

4.6.1. The Human AR Transcriptional Activation Assay for Detection of Androgen (Ant)agonist-Activity of Chemicals (AR STTA) (GD No. 150 Section Annex 2.1)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows antagonism of AR in reporter gene assays, while existing *in vitro* data show a variety of responses including ER antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.6.1.1: Prochloraz data summary

Human AR Transcriptional Activation Assay data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Prochloraz antagonized activation of the reporter gene with R1881. IC50 was approximately 10µM. No agonism was detected. (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	AR binding (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et*	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest	

Human AR Transcriptional Activation Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	al, 2007).	dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-Reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.6.1.2: Conclusions about Human AR Transcriptional Activation Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Prochloraz antagonizes AR combined with effects on ER, S (and possibly T). Potential for adverse effects via multiple mechanisms	Perform assay from upper levels *e.g.* H assay (level 3)	H assay : positive AR antagonism. Reductions in SAT weights at 50 mg/kg/day and above (no effects at 25 mg/kg/day). (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004).
	or fish screen (AFSS) (level 3)	Not available.
	or male PP assay (level 4)	Male PP assay : positive AR antagonism & S disruption. PPS was delayed and SAT weights were reduced at 62 mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).
	or ext-1 or 2-gen assays (level 5)	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).
	or partial/full fish life cycle tests (level 4/5).	FSDT and MMGT: both positive ♀VTG↓; Proportion ♀ ↓; ♂Anal fin papillae ↓; Fecundity ↓ (suggest AR and ER antagonism & S disruption). (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; USEPA 2011, unpublished data)

Table 4.6.1.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	**Conclusions for Prochloraz**
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) study on prochloraz is inadequate for assessment (Cozens *et al*, 1982) but literature pre/post natal studies provide sufficient information to conclude evidence of concern for ED (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b). If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention).
If existing data are from level 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment.	Male PP assay alone (level 4) on prochloraz would not provide sufficient data for ED assessment but would when combined with adequate level 5 (or literature pre & post natal studies).
If existing data are from H assay or AFSS then level 4 mammalian assay or fish screen (TG 229/230) will provide data on multiple modalities.	H assay on prochloraz demonstrates AR antagonism *in vivo*. FSDT and MMGT suggest AR and ER antagonism & S disruption.
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Prochloraz given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides.
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	The prochloraz metabolites 2,4,6-trichlorophenoxyacetic acid and 2,4,6-trichlorophenol had no effect in the AR STTA assay whilst prochloraz was an antagonist. Indicates detoxification by metabolism.

29. Overall conclusions about Human AR Transcriptional Activation Assay and existing data: Next steps and other considerations provide a logical course of action to follow. 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 positive result in the AR STTA assay was supported by a positive result for antagonism in the H assay. The positive results in the male PP assay and level 4 fish assays are also indicative of effects on A, E and S modalities.

1.6. Prochloraz Case Study Results: Wildlife *In vivo* Assays That Have Not Yet Completed Validation

4.7.1 Fish (Medaka) Multi-Generation Test (MMGT) (GD No. 150 Section Annex 2.2)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows depressed fecundity, ♀VTG and ♂ secondary sexual characteristics, while existing *in vitro* data show a variety of responses including aromatase inhibition and steroidogenesis disruption, and existing *in vivo* data show a variety of endpoints consistent with interference with steroidogenesis.

Table 4.7.1.1: Prochloraz data summary

Fish (Medaka) Multi-Generation Test data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
<u>Sub-adults (8 wk pf):</u> F1 ♂Anal fin papillae ↓ (NOEC = 5 µg/L). ♀VTG ↓ (NOEC = 5 µg/L). F2 ♂Anal fin papillae (NOEC = 17 µg/L). ♀VTG ↓ (NOEC = 5 µg/L). <u>Adults (16 wk pf):</u> F0 Fecundity ↓ (NOEC = 25 µg/L) F1 Fecundity (NOEC > 25 µg/L) F2 Fecundity ↓ (NOEC = 17 µg/L) (USEPA 2011, unpublished data)	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	TG 229: NOECs for effects on VTG = <20-30 µg/L, on secondary sexual characteristics = 34 µg/L, and on fecundity = 16-100 µg/L. (Ankley *et al*. 2005; Biever *et al*., 2007; Jensen & Ankley, 2006)
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	TG 230: ♀VTG↓ in 2 fish species (NOEC = 38-58 µg/L) (OECD, 2006)
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010;	TG 416- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75	FSDT: ♀ VTG ↓ (NOEC = 15-64 µg/L) Proportion of ♂ and intersexes ↑ (NOEC = 44-64 µg/L) Proportion ♀ ↓ (NOEC = 44-101

Fish (Medaka) Multi-Generation Test data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Vinggaard *et al* 1999).	mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	µg/L. (Kinnberg *et al*., 2007; OECD, 2007; Katsiadaki pers. comm., 2011; Holbech *et al*., 2011)
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	Non-standard short-term studies with several fish species show effects on various endocrine-related endpoints at concentrations ranging from 8 to 300 µg/L. These are consistent with the results of TG 230. (Ankley *et al*., 2009; Le Gac *et al*., 2001; Liu *et al*., 2011; Skolness *et al*., 2011; Zhang *et al*., 2008)
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)	TG 441 (H assay) - AR antagonism. Reductions in SAT weights at 50 mg/kg/day and above (no effects at 25 mg/kg/day). (Blystone *et al*, 2007; Vinggaard *et al*, 2002; Vinggaard *et al*, 2005a; Birkhoj *et al*, 2004).	
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		A non-standard short-term study with frog larvae revealed an increased proportion of males at 115 µg/L. These are also consistent with the results of TG 230. (Brande-Lavridsen *et*
	5α-Reductase inhibition Lo *et al*, 2007).	Male pubertal assay - PPS was delayed and SAT weights were reduced at 62	
	Inhibition of cortisol secretion		

Fish (Medaka) Multi-Generation Test data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	(Ohlsson *et al*, 2009; Ulleras *et al*, 2008).	mg/kg/day but not at 31 mg/kg/day. Serum T was reduced whilst P and hydroxy-P were increased. NOAEL was 7.8 mg/kg/day based on reductions in serum T at 15 mg/kg/day. (Blystone *et al*, 2007).	*al*. 2008)

Table 4.7.1.2: Conclusions about Fish (Medaka) Multi-Generation Test and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Strong evidence for adverse effects in fish and other organisms by an endocrine mechanism.	Probably no need for additional data.	-

Table 4.7.1.3: Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other considerations (as given in BB)	Conclusions for Prochloraz
None	-

30. Overall conclusions about Fish (Medaka) Multi-Generation Test and existing data: The conclusion that no further action is needed seems justified. Furthermore, the MMGT data suggests that more potent effects do not occur if exposure is extended to the F2 generation. The available data are fully applicable to risk assessment for fish populations, but insufficient data exist for amphibian risk assessments, although the short-term data suggest that amphibian larvae are no more sensitive than fish. No data at all exist to support risk assessment for birds or reptiles.

4.7.2 Larval amphibian growth and development assay (LAGDA) (GD No. 150 Section Annex 2.3)

There are no data available.

4.7.3 Avian two generation test (ATGT) (GD No. 150 Section Annex 2.4)

There are no data available.

1.7. Prochloraz Case Study Results: *In vivo* Mammalian Assays that Have Not Yet Completed Validation Or Not Primarily Designed For Detection Of Endocrine Disruption

4.8.1 Adult male assay (GD No. 150 Section Annex 2.5)

There are no data available

4.8.2 TG 408: Repeated Dose 90 Day Oral Toxicity Study in Rodents (GD No. 150 Section Annex 2.6)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that prochloraz produced positive results on endocrine endpoints in the male rat in a TG 408 assay, the data are indicative of AR antagonism. Existing *in vitro* data show a variety of responses including AR and ER antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.8.2.1: Prochloraz data summary

Repeated Dose 90 Day Oral Rodent Toxicity Study data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Although this is an old study (reported in 1980) prochloraz appears to have had positive results on apical endpoints. Indicators of hormonal activity were not determined. In mice prostate, seminal vesicles ad ovaries were	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 416 (old version)- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement of sensitive endocrine	

Repeated Dose 90 Day Oral Rodent Toxicity Study data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
described as "small" after prochloraz was administered orally at 100 and 400 mg/kg/day. NOAEL was 6 mg/kg/day based on effects in liver. In rats prostate & seminal vesicles were "small", but ovaries were "larger" after prochloraz was administered orally at 100 mg/kg/day. Thyroid weights were increased in ♀ at 6 & 100 mg/kg/day A NOAEL was not identified. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).		endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 453- no ED effects were noted in mice (highest dose was 150 mg/kg/day and or rats (highest dose was 28 mg/kg/day). Liver tumours were increased in mice. (Colley *et al*, 1983; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	Pre/postnatal dosing (250 - 50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).		
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)		
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-Reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion		

Repeated Dose 90 Day Oral Rodent Toxicity Study data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	(Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.8.2.2: Conclusions about Repeated Dose 90 Day Oral Rodent Toxicity Study and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Increased evidence that prochloraz has anti-A activity, S disruption and possibly T disruption.	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).

Table 4.8.2.3 : Other considerations and conclusions for prochloraz. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other Considerations (as given in BB)	Conclusions for Prochloraz
If existing data are from level 5 then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The TG 416 (old version) assay on prochloraz is not to current standards (Cozens *et al*, 1982) therefore would not provide sufficient data for assessment of endocrine disruption by itself. When combined with high quality literature pre & post natal studies (Blystone *et al*, 2007 *etc*) there is sufficient evidence of concern for endocrine disruption. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes more endocrine-sensitive endpoints than TG 416 (*e.g.* AGD in F1 and nipple retention).
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Prochloraz was given orally in all studies. Received dosage partly explains the differences in effects between pre/postnatal studies and old TG

Other Considerations (as given in BB)	Conclusions for Prochloraz
	416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).

31. Overall conclusions about Repeated Dose 90 Day Oral Rodent Toxicity Study and existing data: next steps and other considerations provide a logical course of action to follow. 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 TG 408, as an apical assay, has only a limited ability to discern mechanism but the positive result for antagonism in the H assay indicates that the effects on SATs may be a consequence of this mode of action. These effects also give cause for concern in other wildlife species although the physiological consequences of the effects are likely to be different.

4.8.3. OECD TG 451-3: Combined Chronic Toxicity/Carcinogenicity Studies (GD No. 150 Section Annex 2.7)

Scenario J : Result -, existing *in vitro* data +, existing *in vivo* data +

Scenario J is appropriate because the assay indicates that prochloraz had no effects on endocrine endpoints in carcinogenicity assays. Existing *in vitro* data show a variety of responses including AR and ER antagonism, AR binding, aromatase inhibition, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data show positive results in a variety of endpoints consistent with effects on E A and S modalities.

Table 4.8.3.1: Prochloraz data summary

Combined Chronic Toxicity/Carcinogenicity Assay data for prochloraz	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Prochloraz had no effects on endocrine endpoints. Prochloraz was administered orally up to 150 mg/kg/day in mice and 28 mg/kg/day in rats. Mice developed liver tumours (NOAEL 7.5 mg/kg/day) but there were no treatment-related tumours in rats. No non-neoplastic effects reported on endocrine organs in either species. (Colley *et al*, 1983; as reported in JMPR, 2001).	AR binding. (Noriega *et al*, 2005; Freyberger *et al*, 2010a).	TG 407- reduced SAT weights in rats (♂) at 100 and 400 mg/kg/day. (Lancaster & Shaw, 1980; as reported in JMPR, 2001).	No data
	AR antagonism but no agonism (AR STTA). (Andersen *et al*, 2002; Birkhoj *et al*, 2004; Blake *et al*, 2010; Freyberger *et al*, 2010b; Kjaerstad *et al*, 2010; Noriega *et al*, 2005; Van der Burg *et al*, 2010; Vinggaard *et al*, 2002).	TG 408- reduced SAT weights (♂) and reduced ovary weight at 100 & 400 mg/kg in mice. In rats, reduced SAT weights (♂) and increased ovary weight at 100 & 400 mg/kg/day. Thyroid weight was increased (♀) at 6 & 100 mg/kg/day. (Gale, 1980; Shaw, 1979; as reported in JMPR, 2001).	
	ER antagonism but no agonism (ER STTA, MCF7 cell proliferation assay). (Andersen *et al*, 2002; Bonefeld-Jorgensen *et al*, 2005; Kjaerstad *et al*, 2010; Vinggaard *et al* 1999).	TG 416 (old version)- no obvious endocrine or reproductive effects noted at doses up to 57-75 mg/kg/day. Systemic toxicity occurred at highest dose (75 mg/kg/day) where dystocia and pup deaths were noted. Age of study precluded measurement	

Combined Chronic Toxicity/Carcinogenicity Assay data for prochloraz	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		of sensitive endocrine endpoints. (Cozens *et al*, 1982; as reported in JMPR, 2001).	
	Steroidogenesis disruption (H295R assay). (Hecker *et al*, 2006; Kjaerstad *et al*, 2010; Laier *et al*, 2006; OECD, 2010; Villeneuve *et al*, 2007).	Pre/postnatal dosing (250 -50 mg/kg) resulted in malformed genitalia (♂), altered AGD (♀♂), caused nipple retention (♂), decreased serum T (♂), reduced SAT weight (♂), caused gene expression changes, sweet preference increased (♂). (Blystone *et al*, 2007; Christiansen *et al*, 2009; Laier *et al*, 2006; Noriega *et al*, 2005; Vinggaard *et al*, 2005b).	
	Aromatase inhibition. (USEPA, 2007; Vinggaard *et al*, 2000; Andersen *et al*, 2002; Heneweer *et al*, 2004; Laville *et al*, 2006; Sanderson *et al*, 2002; Trosken *et al*, 2004; Trosken *et al*, 2006).		
	Inhibition of T3-dependent cell growth (thyroid assay) (Ghisari and Bonefeld-Jorgensen , 2005)		
	Possible AhR agonism. (Long *et al*, 2003; Takeuchi *et al*, 2008).		
	5α-Reductase inhibition Lo *et al*, 2007).		
	Inhibition of cortisol secretion (Ohlsson *et al*, 2009; Ulleras *et al*, 2008).		

Table 4.8.3.2: Conclusions about Combined Chronic Toxicity/Carcinogenicity Assay and existing data, and next steps; "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with prochloraz for the suggested assay is shown if available.

Conclusions for Prochloraz	Next Step (as BB)	Assay result for Prochloraz
Prochloraz treatment produced no evidence of adverse effects (neoplastic or non-neoplastic) on endocrine organs in TG 451-3. The multitude of effects seen in level 3 and 4 studies (H and PP assays) and detailed pre and postnatal mechanistic studies indicate that there is evidence of concern for endocrine disruption with prochloraz (Blystone *et al*, 2007 *etc*) Doseage differences may explain some of the differences from existing *in vitro* and *in vivo* data. In rats SAT weights were reduced in the PP assay at 62.5 mg/kg (Blystone *et al*, 2007) whereas the maximum dose in TG 453 was 2-fold lower. Prochloraz does have other effects but dosage difference is the most likely explanation for the lack of non-neoplastic effects observed.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system (level 2). OR Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	Not required as sufficient data are already available. Ext-1 or 2-gen: none to current standards Existing 2-gen study: negative. Age of study precluded measurement of sensitive endocrine endpoints but dystocia was noted. (Cozens *et al*, 1982; as reported in JMPR, 2001).

Table 4.8.3.3 : Other considerations and conclusions for prochloraz; "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for prochloraz.

Other Considerations (as given in BB)	Conclusions for Prochloraz
If existing data are from an adequate level 5 assay then question why differences.	Differences are most likely due to considerations of relative sensitivity. Existing data are from levels 3 & 4. In addition detailed pre and postnatal literature studies provide sufficient evidence that prochloraz poses concern for endocrine disruption (Blystone *et al*, 2007; Christiansen *et al*, 2009, Laier *et al*, Vinggaard *et al*).
Effects seen in existing studies may be in a more sensitive life stage.	Pre and postnatal literature studies on prochloraz provide a more sensitive life stage Blystone *et al*, 2007 etc).
Consider route of exposures for effects data and	Prochloraz was given orally in all studies.

Other Considerations (as given in BB)	Conclusions for Prochloraz
possible implications of ADME characteristics of the chemical	Received dosage partly explains the differences in effects between pre/postnatal studies and old TG 416 study. Prochloraz is rapidly and extensively metabolised to glucuronides (Laignelet *et al* 1989, 1992; Needham *et al* 1991, 1992).
Further mechanistic studies would help determine MoA.	Not necessary as adequate mechanistic data exist.

32. Overall conclusions about Combined Chronic Toxicity/Carcinogenicity Assay and existing data: next steps and other considerations provide a logical course of action to follow. Although the Combined Chronic Toxicity/Carcinogenicity Assay was negative for endocrine effects, the combined dataset dataset indicates that the ER and AR antagonism and S disruption shown *in vitro* also occur *in vivo* in mammals and fish. These effects also give cause for concern in other wildlife species although the physiological consequences of the effects are likely to be different.

33.

4.8.4 OECD TG 421 Reproduction/Developmental Toxicity Screening Test and TG 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (GD No. 150 Section Annex 2.8)

There are no data available

4.9 Prochloraz Case Study: Conclusions

4.9.1 *In vitro* Assays

34. Prochloraz data exist for all of the *in vitro* tests under consideration with the exception of the ER binding assay. All of the assays were positive and were combined with existing positive *in vitro* and *in vivo* data of concern so that Scenario A was considered in all cases. The assay results provided useful information for both mammals and wildlife and the "next steps" and "other considerations" as given in the building blocks appeared logical and useful when tested with the prochloraz data. In all cases it was considered that further *in vivo* testing was not justified as such a wealth of data was already available, even when artificially restricted to that which would be expected for a pesticide. In practice this means that the UT assay was not recommended for testing for prochloraz ER antagonism *in vivo* even though this was detected as a mode of action *in vitro*, as positive higher level apical tests are available. It is considered that the existence of level 4 male PP studies plus detailed pre and postnatal mechanistic studies provides sufficient evidence of endocrine activity without the need for further *in vivo* studies.

4.9.2 *In vivo* Wildlife Assays

35. There are a number of gaps in the wildlife dataset, although there are enough data to permit an evaluation of the fish-based building blocks. Apart from the lack of avian and amphibian data, the most important data-gap is the lack of an Androgenised Female Stickleback Screen (AFSS) which would be expected to be highly sensitive to the anti-androgenic action of prochloraz. The non-standard data available in the published literature generally support the results from standardised tests.

36. The building blocks providing advice on the wildlife assays for which data exist generally produce logical outcomes when compared with the results of higher level tests, and especially when one considers data from the Medaka Multi-Generation Test (MMGT). There are no instances of a lower level wildlife assay giving a false negative when compared with higher level wildlife data, neither are there any false positives. The TG 229 and 230 *in vivo* fish screens appear to be of broadly similar sensitivity to prochloraz, although one TG 229 NOEC (for fecundity) appeared to be somewhat lower than the TG 230 NOECs (for VTG). Interestingly, the sensitivity of the apical endpoints of TG 229, TG 234 and the MMGT are comparable, with NOECs ranging from 16 to 100 µg/l. Furthermore, although no data from a standardised amphibian assay are available, a non-standard test with frog larvae (Brande-Lavridsen *et al*. 2008) suggests that there may be scope for extrapolating the effects of prochloraz from fish to amphibians. Finally, the F0 and F1 generations in the MMGT do not appear to be substantially less sensitive to prochloraz than the F2 generation, but no data from a Fish Full Lifecycle Test (FLCTT) are available to support or refute this conclusion. Furthermore, it is possible that some chemicals would produce larger effects on the F2 generation than in the F0 or F1 generations, even though NOECs may be similar.

37. Unfortunately, there are no available avian data, or higher tier amphibian data, so the potential for extrapolating from fish to birds cannot be evaluated, and the scope for extrapolating to amphibians (as suggested by the data of Brande-Lavridsen *et al*. 2008) cannot be examined further at present.

4.9.3 *In vivo* Mammalian Assays

38. There are data available for prochloraz for most of the mammalian assays considered in the GD. The exceptions are the UT assay, female PP assay, extended 1 generation assay (TG 443) and TG 421/422. The endocrine-specific assays from the CF have generally been conducted recently and therefore contain endocrine disruption-sensitive endpoints. On the other hand, the standard toxicology tests conducted for

pesticide registration were generally conducted in the 1980s and therefore are not to modern standards. It is notable however, that the tests with designs equivalent to TG 407 and TG 408 (28 and 90 day toxicity studies respectively) gave positive results of reductions in male reproductive organ weights (SATs) in accordance with the recent Hershberger and male PP assays. Increases in thyroid weight also occurred in the 90 day toxicity study.

39. A 2-generation study and a carcinogenicity study (also conducted in the 1980s) both had no effects on any endocrine endpoints. The absence of effects in these studies is likely due to both doseage considerations (the doses employed were lower than in the tests where positive results were obtained) and an absence of endpoints sensitive to endocrine disruption.

40. The endocrine-specific assays from the CF were positive: H assay (level 3) and male PP assay (level 4). The negative result in the (old) TG 416 assay has been superseded for hazard (and risk) determination purposes by the existence of a number of excellent pre and postnatal mechanistic studies. These demonstrate endocrine-related developmental effects in rats and effectively provide higher tier data. It is likely that if an extended-1-generation study were to be conducted on prochloraz, the endocrine-sensitive endpoints would be significantly altered.

41. The combination of negative and positive results, however, meant that scenarios A and J were tested with prochloraz. In all cases, the guidance provided seemed logical and helpful. The consideration of all the available data (allowing for the restrictions we placed in not considering higher CF level data for lower CF level tests in order to mimic a realistic situation) allowed us to conclude in every case that further *in vivo* testing was not warranted. Ultimately, at the highest level, the combined dataset allowed an estimation of NOAELs for development and reproduction.

4.9.4 Overall Conclusions About Prochloraz

42. In summary, although data on prochloraz from a number of assays are unavailable, this case study suggests that the evaluated building blocks generally provide sound advice about data interpretation and possible next steps. It also seems likely that more restricted 'Existing Data' would not lead to substantially different conclusions, although this was not formally evaluated.

2. 4-Tert-Octylphenol Case Study Results

5.1 Data used in the 4-tert-Octylphenol Case Study Analysis

43. There are a large number of studies relevant to the potential endocrine modulating effects of tert-OP and it was not considered necessary or appropriate for this case study to list them all. A complete listing has recently been carried out during the compilation of a dossier to consider whether tert-OP should be considered an SVHC (BauA, 2011). There are numerous duplicative studies of ER binding, ER-transactivation assays and uterotrophic assays. In this case study a few representative, recent studies have been selected. In addition, repeat dose studies using intraperitoneal dosing or where substantial systemic toxicity has occurred have also been excluded. Data for standardised assays used in the case study on tert-OP for GD No. 150 are given in Table 5.1.1. The format of the table follows that given in the Contents of

the GD so that the two documents can be easily compared. Data, from non-standardised assays, considered relevant for assessment of endocrine effects are given in Table 5.1.2. These data do not comply with the Test Guideline study designs in the CF but are considered to be of relevance for assessment of effects on the health of humans or wildlife.

Table 5.1.1: Data for standardised assays used in the case study on tert-OP for the OECD GD on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (No. GD 150). Note that the "Result" column indicates a positive (+), negative (-) or equivocal (Eq) result for endocrine endpoints only.

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
	A. Validated assays for which guidance is provided in the main GD			
	***In vitro* Screens**			
C.2.1	ER Binding Assay (US EPA OPPTS 890.1250)		**Standard Assay:**	
		+	Rat uterine cytosol. Tert-OP displaced E2 (Ki=0.781 μM, IC50=2.08 μM).	Laws *et al* (2000)
		+	Rat uterine cytosol. Tert-OP displaced E2 (Ki=37.7 μM, IC50=12 μM).	Laws *et al* (2006)
			Non-Standard Assay:	
		+	Cytosol from endometrial adenocarcinoma cell line (RUCA-I). Tert-OP displaced E2, magnitude of response relative to E2 was 0.013% .	Strunck *et al* (2000)
		+	Mouse uterine cytosol. Tert-OP displaced E2 (EC50=0.16 μM).	Yoon *et al* (2000)
		+	hER extracted from MCF-7 cells. Tert-OP displaced E2 (IC50=38 μM).	Olsen *et al* (2005)
		+	Purified bacterially expressed hERα/β. Tert-OP displaced E2. ERα relative binding affinity = 0.013, ERβ relative binding	Routledge *et al* (2000)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			affinity = 0.25.	
		+	Cloned hERα/β. Tert-OP displaced E2. ERα IC50=2.08 μM, ERβ IC50=3.52 μM.	Sahambi *et al* (2010)
C.2.2	AR Binding Assay (US EPA OPPTS 890.1150)		**Non-standard Assay:**	
		+	PALM cells expressing hAR. Tert-OP displaced R1881 (IC50=3 μM, Ki=25 μM).	Paris *et al* (2002)
		+	Yeast cells expressing hAR. Tert-OP displaced R1881 (IC50=1.6 μM)	Li *et al* (2010)
C.2.3	OECD TG 455: The Stably Transfected Human ERα Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA) (including Guidance for the Antagonism Assay)		**Standard Assay:**	
		+	HeLa cells with luciferase as reporter gene. Tert-OP was agonistic (EC50=0.1 μM)	OECD (2006b)
			Non-standard Assay:	
		+	Transient transfection assay. HeLa cells with luciferase as reporter gene. Tert-OP was agonistic (PC10=0.12 μM), but not antagonistic with E2.	Yamasaki *et al* (2002)
		+	MCF-7 cells with luciferase as reporter gene. Tert-OP was agonistic (EC50=19 μM) and antagonistic with E2 (LOEC=28 μM).	Ghisari *et al* (2009)
		+	MCF-7 cells with luciferase as reporter gene. Tert-OP was agonistic (EC50=12.5 μM).	Wu and Safe (2007)
		+	Yeast cells with β-gal as reporter gene. Tert-OP was agonistic (EC50=0.17mg/L).	Isidori *et al* (2006)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
C.2.4	OECD TG 456: H295R Steroidogenesis Assay		**Standard Assay:** No data available	
		+	**Non-standard Assay:** The effect of tert-OP on testosterone biosynthesis/testicular steroidogenic competence was investigated *in vitro* in adult rat Leydig cells. Exposure to tert-OP at 0.5 to 2 µM for 4 or 24 h increased testosterone levels (up to 2-fold in 4-h exposed cells). Subsequent studies have indicated that the effects are not mediated via ER.	Murono *et al* (2000, 2001, 2002)
		+	The effect of tert-OP on testosterone and progesterone production was investigated *in vitro* in fetal rat testes in culture. Significantly increased testosterone and progesterone levels occurred at 10, 100, 500 mg/L. E2 levels were unchanged.	Haavisto *et al* (2003)
C.2.5	Aromatase Assay (US EPA OPPTS 890.1200)		No data available	
	Wildlife Screens and Tests			
C.3.1	OECD TG 229: Fish Short Term Reproduction Assay (FSTRA)		**Standard assay:** Exposure range = 0.6-113 µg/l	Biever *et al*. 2007
		+	**Plasma VTG** was induced in male fathead minnow (*Pimephales promelas*) with a LOEC of 0.6-37 µg/l.	
		+	Male **secondary sexual characteristics** were reduced with LOECs of 0.6->113 µg/l.	
			Fecundity was reduced with	

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
		+	LOECs of 113 µg/l.	
		+	**Fertility** was reduced with a LOEC of 113 µg/l.	
			Non-standard assay:	
		+	Adult male medaka (*Oryzias latipes*) were exposed to tert-OP for 21 d at measured concentrations between 20 and 230 µg/l. After exposure, males were mated with unexposed females. **Plasma VTG** was elevated in males (NOEC = 20 µg/l)	Gronen *et al*. 1999
		+	**Fecundity** reduced by ~50% after matings with males exposed at all concentrations (NOEC < 20 µg/l). % fertilisation was also reduced, but not significantly.	
C.3.2	OECD TG 230: 21 Day Fish Assay		**Standard assay:**	
		+	Concentration range = 13-296 µg/l **Plasma VTG** in male medaka (*Oryzias latipes*) was elevated by a factor of approx. 50 at the LOEC of 64 µg/l (NOEC = 27.8 µg/l).	Japanese Ministry of the Environment 2006
			Non-standard assay:	
		+	3-month old male and female medaka (*Oryzias latipes*) were exposed to tert-OP for 21 d in separate containers at measured concentrations ranging from 12.7 to 296 µg/l. **Hepatic VTG** was induced with a 21 d NOEC in males of 27.8 µg tert-OP/l, and in females of 129	Nozaka *et al*. 2004

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			µg tert-OP/l. VTG levels in liver were 2-fold higher in males than females, with a maximum concentration of approx. 8000 ng/mg liver at the highest tert-OP concentration.	
C.3.3	OECD GD 140: Variant of OECD TG 230: Androgenised Female Stickleback Screen (AFSS)		No data	
C.3.4	OECD TG 234: Fish Sexual Development Test (FSDT)	+	**Standard assay:** Concentration range = 10-320 µg/l	OECD 2011
		+	NOECs for **VTG induction** in zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and stickleback (*Gasterosteus aculeatus*) were 9.5-17.6 µg/l, 6.2-31.7 µg/l, and 22.2->41.9, respectively. NOECs for **altered sex ratio** (excess ♀) in the same species were 5.7-<13.8 µg/l, <11.2-23.5 µg/l, and >41.9-66 µg/l, respectively.	
		+	**Non-standard assay:** Medaka (*Oryzias latipes*) were continuously exposed to tert-OP at 2, 20 and 50 µg/l from 2-4 h post-fertilisation until maturity. 20 and 50 µg/l caused a 20-30% increase in mortality before and after hatch, and **growth** was reduced at 50 µg/l (NOEC = 20 µg/l). **Sex ratio** was biased towards females (42% in controls, 58% in treated fish) at 50 µg/l (NOEC = 20 µg/l), and 3-4% of these fish developed testis-ova. If exposed males were mated with	Knörr & Braunbeck 2002

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			control females, **fertilisation rate** was reduced by up to 11%. When exposed females were mated with control males, mortality of offspring of fish exposed to 20 and 50 µg/l at least doubled (NOEC = 2 µg/l).	
		+ +	Medaka (*Oryzias latipes*) were continuously exposed to tert-OP from fertilisation to 60 d post-hatch at mean measured concentrations of 6.9, 11.4, 23.7, 48.1 and 94.0 µg/l. The NOEC for elevated male **hepatic VTG** was 6.9 µg/l (with an approximate doubling of VTG levels at 11.4 µg/l compared to controls), and for a female-biased **sex ratio** (determined by secondary sex characteristics) was 23.7 µg/l, with 76% phenotypic females at 48.1 µg/l and 100% females at 94 µg/l. At the top concentration, 25% of the phenotypic females were observed to have testicular gonadal tissue, and 25% showed testis-ova. When transferred to clean water, the sex ratio of fish from the top concentration partially returned to normal over a period of 60 d.	Seki *et al*. 2003
C.3.5	Fish Lifecycle Toxicity Test (FLCTT) (USEPA OPPTS 850.1500)	+ +	**Standard assay:** Concentration range = 1.7-82.3 µg/l Medaka (*Oryzias latipes*) in F0 and F1 generations showed **testis-ova** in 50-70% of fish at the LOEC of 30 µg/l (NOEC = 9.9 µg/l). **Male VTG** was induced in F0 and F1 generations by a factor of 7-30	Japanese Ministry of the Environment 2006

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
		-	at the LOEC of 9.9 µg/l (NOEC = 4.3 µg/l). There were no significant effects on **growth**, **GSI** or **time to hatching**.	
		Eq	**Hatchability** of the F1 generation was reduced by half at 82 µg/l, but not significantly.	
			Non-Standard assay:	
		+	Full lifecycle tests starting with fertilised eggs were performed with zebrafish (*Danio rerio*) exposed to tert-OP at measured concentrations of 1.2, 3.7, 11.9 and 38 µg/l. Time to first **spawning** and **fecundity** were both affected, but the most consistent and reproducible effect was a reduction in **fertilisation success**, the EC50 of which was 28 µg/l from day 78 onwards.	Segner *et al*. 2003
		+		
		-	Full lifecycle test starting with fertilised eggs of zebrafish (*Danio rerio*) exposed to measured tert-OP concentrations of 1.2, 3.2, 12 and 35 µg/l. There were no significant effects on **growth or survival** of either the F0 or F1 generations, and no effects on **sex ratio**, with a NOEC of 12 µg/l .	Wenzel *et al*. 2001
		+	There was a significant reduction in **growth**, **fecundity** and **fertility** and a significant increase in **time to first spawning,** with a NOEC of 12 µg/l.	
C.3.6	OECD TG 231: Amphibian Metamorphosis Assay		**Standard assay:**	

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
	(AMA)		No data	
C.3.7	OECD TG 206: Avian Reproduction Test		**Standard assay:** No data	
	Mammalian Screens and Tests			
C.4.1	OECD TG 440: Uterotrophic Bioassay in Rodents (UT assay) (Including GD on the Use of the Assay to Screen for Anti-Estrogenicity)		**Standard Assay:**	
		+	Immature rats were administered tert-OP by gavage or s.c. injection at 0, 50, 100, 200 or 400 mg/kg/d for 3 days (n=6). Uterine weight was increased at 100 mg/kg/day and above by both routes but the magnitude of change was greater after s.c dosing. Ovariectomized rats administered tert-OP by gavage at 0, 50, 100 or 200 mg/kg/day for 3 days had increased uterine weight at 100 and 200 mg/kg/day (n=6).	Laws *et al* (2000)
		+	Ovariectomized rats were administered tert-OP by gavage at 0, 5, 50 or 200 mg/kg/day for 3 days (n=6). Uterine weight was increased at 200 mg/kg/day, NOAEL was 50 mg/kg/day.	Diel *et al* (2000)
		+	Ovariectomized rats were administered tert-OP s.c. at 0, 10, 50, 200 or 400 mg/kg/day for 3 d (n=6). Uterine weight was increased at 50 mg/kg/day and above, NOAEL was 10 mg/kg/day.	Kwack *et al* (2002)
		+	Immature rats were administered tert-OP s.c. at 0, 2, 20 or 200 mg/kg/day for 3 days (n=6). Uterine weight was increased at 200 mg/kg/day and above, NOAEL was 20	Yamasaki *et al* (2002)
		+		Katsuda *et al*

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			mg/kg/day. Ovariectomized rats were administered tert-OP s.c. at 6.25, 12.5, 25, 50, 100 or 200 mg/kg/day for 2 or 14 days. (n=5). Uterine weight was increased at 50 mg/kg/day and above after 2 d of treatment (NOAEL 25 mg/kg/day) and at 25 mg/kg/day after 14 d of treatment (NOAEL 12.5 mg/kg/day).	(2000a)
C.4.2	OECD TG 441: Hershberger Bioassay in Rats (H Assay) (Including OECD GD for Weanling Hershberger Bioassay)	-	**Standard Assay:** Castrated rats were administered tert-OP by gavage at 50, 200 or 600 mg/kg/day for 10 days in the presence and absence of testosterone (n=6). All rats given 600 mg/kg/day died and a significant decrease in body weight was observed at 200 mg/kg/day. No androgen agonistic or antagonistic effects were seen at doses of 50 or 200 mg/kg/day.	Yamasaki *et al* (2003)
C.4.3	Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats (Male PP Assay) (US EPA OPPTS 890.1500)		No data available	
C.4.4	Pubertal Development and Thyroid Function Assay in Peripubertal Female Rats (Female PP Assay) (US EPA OPPTS 890.1450)	+	**Standard Assay:** No data available **Non-standard Assay:** Rats were given tert-OP by gavage at doses of 0, 50, 100 or 200 mg/kg/day from PND 21 to 35. Age of vaginal opening was advanced (by 3.2 days) at 200 mg/kg/day (n=7). Body weight was unaffected. No other	Laws *et al* (2000)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			parameters were determined.	
C.4.5	OECD TG 407: Repeated Dose 28 Day Oral Toxicity Study in Rodents		**Standard Assay:**	
		-	Twenty eight day oral gavage study in BOR Crj:CD (SD) rats (0, 15, 70, 300 mg/kg/day), n=6. Body weight gain was reduced in high dose males. There were no changes in organ weights or histopathology of endocrine or endocrine-responsive organs.	CIPT (1994) (As cited in SIDS, 1994)
		-	Twenty eight day oral gavage study in SD rats (0, 15, 150, 250 mg/kg/day), n=5. There were no effects on body weight. There were no changes in organ weights or histopathology of endocrine or endocrine-responsive organs.	HRC (1994) (As cited in SIDS, 1994)
			Non-standard Assay:	
		+	Intact adult female Long Evans rats (7-14/group) were administered tert-OP by gavage (0, 50, 100 or 200 mg /kg/day). Periods of extended estrus and disturbances in the estrus cycle were observed at 200 mg/kg/day. No other parameters were determined.	Laws *et al* (2000)
		+	Male SD rats were administered tert-OP by gavage at 0, 50, 150 or 450 mg/kg/day for 30 days (n=12). Testicular function was determined. At 450 mg/kg/day only, body weight gain was reduced and some testicular parameters were altered. Weights of testes, epididymis and prostate were reduced and histopathological changes	Bian *et al* (2006)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			occurred. Testicular sperm counts were reduced. Some changes in sperm motility occurred whilst others were unaffected. No effects occurred at 150 or 50 mg/kg/day.	
		+	Male Wistar and Fischer rats were administered tert-OP by gavage at 0, or 400 mg/kg/day for 1 month (n=9-10). Strain differences in sensitivity were determined. Body weights were unaffected by treatment. Relative weights of liver and kidneys were increased in both strains. Relative weight of the adrenals were significantly increased and relative weights of seminal vesicles and the levator ani/bulbocavernosus muscle were significantly reduced in the Fisher strain, whereas in the Wistar strain only the relative weight of the levator ani/bulbocavernosus muscle was decreased. No weight changes in either strain were observed for testes, epididymis or ventral prostate weight. There were no changes in serum testosterone, LH, FSH or inhibin and no changes in testicular histopathology in either strain.	Hossaini *et al* (2003)
		-	Female SD rats were administered tert-OP by gavage at 0, 25, 50 or 125 mg/kg/day for 35-41 days (killed at the same stage of the estrus cycle) (n=7). Effects on ovaries and uterus were examined, There were no effecs on serum E2, organs weights or histopathology or gene expression using microarray analysis.	Sahambi *et al* (2010)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
C.4.6	OECD TG 416: Two-Generation Reproduction Toxicity Study (Including TG 415: One-Generation Reproduction Toxicity Study)	-	**Standard Assay:** Tert-OP was tested in a 2-generation reproduction toxicity study with several enhancements. SD rats (n=30) were fed with dietary concentrations of 0, 0.2, 20, 200, and 2000 ppm 4-tert-octylphenol, leading to a daily intake of 0.011-0.034, 1.05-3.3, 32.6-10.9, and 111-369 mg/kg body weight/day depending on the age and sex of the animals and the phase of the study. The rats were dosed for 10 weeks pre-breeding (F0), then following selection of F1 pups for breeding, a further 10 weeks pre-breeding dosage took place before production of the F2 generation. The F2 pups were also fed tert-OP after weaning. Female F2 rats were killed following vaginal opening. Male F2 rats were maintained through acquisition of preputial separation and until 111 ± 5 days of age to allow for evaluation of sperm parameters. Reductions in body weight and body weight gain occurred in both sexes and all generations at 2000 ppm. There were no treatment-related effects in F0 or F1 females on mating, fertility, pregnancy, gestational indices, number of implants, total pups, live or dead pups per litter, percentage postimplantation loss (prenatal mortality index), or gestational length (in days). Oestrous cycle length in days and stage of oestrus at necropsy were equivalent across all groups. There were no	Tyl *et al* (1999)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			treatment-related effects on reproductive organ or histology. There were no effects of treatment in F0 or F1 males on mating or fertility indices. There were also no treatment-related effects in F0, F1, and F2 males on weights of reproductive organs and no effects on sperm parameters. The ages at acquisition of vaginal patency and preputial separation in F1 and F2 pups at 2000 ppm were marginally but significantly delayed ($P < 0.01$) compared to controls, with effects on body weights at acquisition. The marginal delays (both less than 2 days), and were considered related to the lower body weight, starting late in lactation and continuing through the post wean exposure period. AGD was determined in F2 offspring and was unaffected in male pups. AGD in the newborn F2 females was longer in all tert-OP treated groups: 0.79, 0.81, 0.85 and 0.79 mm compared to 0.76 mm in controls. However, this was within historical control values and was not considered to be biologically significant. **Non-standard Assay:**	
		Eq	Male Crj:Donryu neonatal rats were exposed to 4-tert-octylphenol by s.c. injection of 100 mg/kg/day 24 h after birth. Male pups were then treated every other day until pnd 15 (8 doses). At pnd 6, 10, 14, 21, and 28, and at 5, 7, 8, and 18 weeks of age 4-8	Yoshida *et al* (2001)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			rats per group were randomly selected from different litters and terminated (F0). At 13 weeks of age, males from both the control and the 4-tert-octylphenol treated group were selected and mated with untreated females to examine reproductive ability (n=7). F0 pups were terminated at 18 weeks of age. F0 rats had slight but significantly lower testis, epididymis and prostate weights from 10 days of age until 7 weeks. These changes were not seen at later ages (8-18 weeks). No histopathological changes were seen at any time (including morphometrical stage analysis of testicular spermatogenesis). There were no differences between the control and the treated group in mating success and fertility including average number of live embryos per litter. Epididymal sperm head numbers in F0 rats at 18 weeks, however, were slightly reduced. No differences between groups were found in the area of the SDN-POA or in its length or width. Prepubertal FSH levels were lower in the tert-OP treated group until pnd 14 and increased thereafter. LH levels were unchanged between groups. Testosterone levels in 4-tert-octylphenol treated rats up to 7 weeks of age were lower than in the controls.	
C.4.7	Extended One-Generation Reproductive Toxicity Study		No data available	

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
	(draft OECD TG 443)			
	B. Assays that have not yet completed validation, or have not primarily been designed for detection of endocrine disruption, for which limited guidance is given in Annex 2 of the GD			
	***In vitro* Screens**			
Annex 2.1	The Human AR Transcriptional Activation Assay for Detection of Androgen (Ant)agonist-Activity of Chemicals (AR STTA)	+	CHO cells, transiently transfected with luciferase as reporter gene. Tert-OP was antiandrogenic in the presence of R1881 (IC50=1.2 µM).	Krüger *et al* (2008)
		+	PALM cells(stably transfected, luciferase reporter gene). Tert-OP was antiandrogenic in the presence of R1881 (IC50=3µM, Ki=25µM).	Paris *et al* (2002)
	Wildlife Screens and Tests			
Annex 2.2	Fish (Medaka) Multi-Generation Test (MMGT)		No data available	
Annex 2.3	Larval Amphibian Growth and Development Assay (LAGDA)		**Standard assay:** **Non-standard assay:** Western clawed frog (*Xenopus tropicalis*) were exposed to measured concentrations of tert-OP (1, 3.3, 11 and 36 µg/l) for 31	Porter *et al*. 2011

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			weeks from NF stage 46 larvae.	
		+	The top concentration increased **serum VTG** in male and female larvae by a factor of 3 (NOEC = 11 µg/l) but this effect was not seen in the subsequent adults.	
		+	**Oviducts** were observed to grow and develop in ~20% of male adults (0% in controls) (NOEC = 11 µg/l).	
		-	There were no effects on adult **phenotypic sex ratio**, **sperm or oocyte counts**, or **estradiol or testosterone titres**.	
Annex 2.4	Avian Two Generation Test (ATGT)		**Standard assay:** No data available	
	Mammalian Screens and Tests			
Annex 2.5	Adult Male Assay		No data available	
Annex 2.6	TG 408: Repeated Dose 90 Day Oral Toxicity Study in Rodents	-	**Standard Assay:** Three month oral feeding study in BOR/WISW rats [(30, 300, or 3000 ppm (corresponding to 2.3, 23, 230 mg/kg/day)], n=20. No signs of systemic toxicity. There were no changes in organ weights or histopathology of endocrine organs.	Suberg *et al* (1982)
		-	**Non-Standard Assay:** Male SD rats were administered tert-OP by gavage at 0, 25, 50, or 125 mg/kg/day for 60 days	Gregory *et al* (2009)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			(n=10). Effects on male reproductive organs were determined. Decreased body weight occurred at the highest dose. Some minimal effects on caudal sperm counts were reported but there were no effects on the weights of the testes, epididymides, ventral prostate, and seminal vesicles or on histopathology of testes and epididymides. No effect on testicular gene expression was reported.	
Annex 2.7	OECD TG 453: Combined Chronic Toxicity/Carcinogenicity Studies (including TG 452 Chronic Toxicity Studies)		No data available	
Annex 2.8	OECD TG 421 Reproduction/Developmental Toxicity Screening Test and TG 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test	-	**Standard Assay:** Male and female rats were administered tert-OP at 0, 125, 250, 500 mg/kg/day, orally for 2 weeks prior mating, during 2 weeks mating, until day 4 post partum(6 weeks total) (n=12). Severe systemic toxicity occurred at the highest dose, along with reduced weights of reproductive organs and impaired reproduction but without gross abnormalities in the offspring. At the lower doses there were no effects on endocrine or reproductive organs and no effects on mating performance or litter development.	HRC (1995) (As cited in SIDS, 1994)
		-	**Non-Standard Assay:** Six xenobiotics (including tert-OP were tested using OECD TG 421. At the dose selected (1000 mg/kg/day), tert-OP was lethal	Piersma *et al* (1998a, b)

GD Section	Tests and Screens	Result (+ - Eq)	Data for 4-tert-octylphenol	Reference
			and therefore new rats were enrolled into the study which were dosed from 12 days premating onward, at 100 mg/kg/day. Only 4 rats/group were used. No compound-related effects on fertility, spermatogenesis, fetal development or serum hormone levels (E2, progesterone, testosterone, LH, FSH) were determined	

Table 5.1.2: Data for non-standardised assays used in the case study on tert-OP for GD No. 150. *In vitro* and *in vivo* data are given separately (under the headings in the "Building Blocks" within the GD) and are listed in alphabetical order of author.

Summary of Non-Standard tert-OP Data	**Reference**
Mechanism (*in vitro* mechanistic data)	
Studies using mammalian cells/receptors:	
PR binding assay. Rat uterine cytosol from ovariectomised rats was used. Tert-OP was positive with weak binding affinity (Ki=3.8 μM, IC50=11.4 μM)	Laws *et al* (2000)
Tert-OP exhibited estrogen agonist activity when tested in recombinant yeast transcriptional activation assays expressing ER or AR. ER antagonism, AR agonism and AR antagonism did not occur. The glucuronide metabolite of 4-tert-OP was also tested but showed no activity indicating that this pathway is a detoxification reaction.	Moffat *et al* (2001)
MCF-7 cell proliferation assay (positive for estrogenicity). EC50=10-7 M.	Rajapakse *et al*. (2004)
MCF-7 cell proliferation assay (positive for estrogenicity). EC50=5x10-6 M.	Olsen *et al* (2005)
MCF-7 cell proliferation assay (positive for estrogenicity). EC50=2x10-7 M,	Sahambi *et al* (2010)

Rat pituitary GH3 cell proliferation assay (TH dependent). Tert-OP stimulated cell growth (LOAC 10^{-6} M) and inhibited T3-stimulated cell growth (LOAC 10^{-5} M)	Ghisari *et al* (2009)
H295R assay. Dose-dependent reductions in cortisol secretion occurred. 4-tert-OP strongly inhibited CYP11A activity at 12.5 and 25 µM, and inhibited CYP17 and CYP21B at 25 µM. No effects were seen on 3ß-HSDII activity.	Nakajin *et al* (2001)
Primary bovine oocytes were cultivated *in vitro* with 1 to 0.0001 µg tert-OP/ml. Oocyte maturation and fertilization were decreased. Oocyte ERα was decreased, but ERß and PR mRNA were not affected.	Pocar *et al* (2003)
Rat ovarian cells were cultured with 4-tert-octylphenol (up to 1000 nM). There were no effects on progesterone production.	Akgul *et al* (2008)
Ovarian follicles from 14 day old rats were cultured with 4-tert-octylphenol. Concentration-dependent inhibition of E2 and testosterone secretion occurred at 10-8, 10-7, 10-6 M). There were no effects on aromatase activity, but forskolin-induced cAMP levels were decreased.	Myllymäki *et al* (2005a)
Ovarian follicles from 14 day old rats were cultured with 4-tert-octylphenol after treatment with 4-tert-octylphenol on pnds 6, 8, 10, and 12. E2, testosterone and progesterone and cAMP production were decreased.	Myllymäki *et al* (2005b)
Studies using non-mammalian cells/receptors:	
In male eelpout (*Zoarces viviparus*), 48 h exposure of the fish to tert-OP at 10 mg/kg reduced E2 affinity for the ER in hepatic cytosolic extracts and upregulated the number of E2 binding sites. In females, the EC50 for ER-binding of tert-OP was 1215 µg/l, with a relative binding affinity compared with E2 of 0.0011.	Andreassen & Korsgaard 2000
The binding affinity of tert-OP for estrogen receptors from the liver of rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*) was measured. IC50 values were 37 µg/l for fathead minnow and 148,550 µg/l for rainbow trout, and the relative binding affinities with respect to E2 were 0.013 and 0.009, respectively.	Denny *et al*. 2005
Estrogen receptor alpha and beta were cloned from the cyprinid fish *Varicorhinus barbatulus* and used to test the activity of tert-OP in a yeast-based reporter system. tert-OP produced dose-dependent responses in ligand with ER alpha exhibiting higher magnitude of responses than ER beta.	Fu *et al*. 2007 [paper not seen]
COS-7 cells were transiently transfected with ER α and β from channel catfish (*Ictalurus punctatus*) and the ability of tert-OP to displace estradiol (E2) from the receptors was measured. tert-OP showed weak relative binding affinity for ER α (0.16) and ER β (0.01) compared with E2.	Gale *et al*. 2004
A primary culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes was used to measure the estrogenicity of tert-OP through induction of VTG. The EC50 for	Jobling & Sumpter 1993

VTG induction was 435 µg/l, and the relative magnitude of response with respect to E2 was 0.000037.	
2 breast cancer cell lines (ZR-75 and MCF-7), were used to test tert-OP. An tert-OP concentration of 2063 µg/l caused a quadrupling of breast cancer cell growth, almost as great as that caused by E2, and this concentration produced maximal transcriptional activity of the ER, with some activity still evident at 20 µg/l.	Jobling *et al*. 1995
A clawed frog (*Xenopus laevis*) liver cytosolic preparation was used to test the competitive binding affinity of tert-OP to the ER. Receptor binding of tert-OP was measurable (IC50 = 16.1 mg/l), and was relatively weak compared with E2 (relative magnitude of response = 0.00054) and a range of environmental chemicals.	Lutz & Kloas 1999
Channel catfish (*Ictalurus punctatus*) hepatocytes were exposed to tert-OP at 2.1, 206 or 2060 µg/l for 4-6 d. All concentrations induced VTG over the range 65-303 ng VTG/ml.	Monteverdi & Di Giulio 1999
Rainbow trout (*Oncorhynchus mykiss*) hepatocytes were exposed to tert-OP at 0.21-2060 µg/l, giving a VTG response at 206 µg/l, with cytotoxicity at 2060 µg/l. Magnitude of response relative to E2 was 0.000032. The IC50 for ER-binding was 173 mg/l.	Olsen *et al*. 2005
A rainbow trout (*Oncorhynchus mykiss*) hepatocyte culture was used to test the estrogenicity of tert-OP through measurement of VTG production. The 96 h EC50 for VTG production was 2785 µg/l, but some cytotoxicity was evident.	Petersen & Tollefsen 2011
Primary cultures of hepatocytes from the Chinese minnow (*Rhynchocypris oxycephalus*) were used to measure the estrogenicity of tert-OP through induction of VTG. VTG was induced at a concentration of 20.6 mg/l.	Park *et al*. 2003 [paper not seen]
A rainbow trout (*Oncorhynchus mykiss*) hepatocyte culture was used to test the estrogenicity of tert-OP through measurement of VTG production. The 3 d EC50 was 8528 µg/l, and magnitude of response relative to E2 was 0.00083. Carp (*Cyprinus carpio*) hepatocytes were exposed to tert-OP and VTG was induced with a 3d EC50 of 7868 µg/l. The relative magnitude of response compared with E2 was 0.0031.	Segner *et al*. 2003
A primary culture of hepatocytes from Atlantic salmon (*Salmo salar*) was used to measure the estrogenicity of tert-OP through induction of VTG. The EC50 for VTG induction was 59.7 µg/l, with a relative magnitude of response compared with E2 of 0.0091.	Tollefsen *et al*. 2003
A primary culture of hepatocytes from the brown bullhead (*Ameriurus nebulosus*) was exposed to tert-OP and VTG induction used to measure estrogenicity. VTG was induced at all concentrations tested (2-20 mg/l), by up to a factor of 70, and the effect could be antagonised by the anti-estrogen tamoxifen.	Toomey *et al*. 1999
A variety of *in vitro* assays (VTG induction in rainbow trout *Oncorhynchus mykiss* hepatocytes; gene transcription in cells transfected with the ER; growth of breast cancer cell lines) were used to show that tert-OP (20.6-2060 µg/l) was able to stimulate responses of similar magnitude to E2, although at concentrations	White *et al*. 1994

1000-fold higher. The lowest concentration tested (20.6 µg/l) caused VTG induction.	
Effects (*in vivo* effects of concern) **<u>Studies in wildlife species : Fish</u>**	
Male eelpout (*Zoarces viviparus*) were exposed to tert-OP by intra-peritoneal injection at 10 mg/kg on days 2 and 14. This caused significant increases in plasma VTG at both exposure times.	Andreassen & Korsgaard 2000
Male eelpout (*Zoarces viviparus*) were exposed to tert-OP at 25 µg/l. After 48 h, hepatic estrogen receptor (ER) transcripts were induced 6-fold, and these had increased to 20-fold after 1 week. Vitellogenin (VTG) transcripts in blood plasma were also induced later in the timecourse.	Andreassen *et al*. 2005
tert-OP at nominal concentrations of 1-50 µg/l reduced growth (up to 50%) at 108 d in juvenile female rainbow trout (*Oncorhynchus mykiss*) after a 22 d exposure. However, no effects were observed on ovosomatic index at 466 d after a 35 d exposure.	Ashfield *et al*. 1998
Salmon parr (*Salmo salar*) were exposed to 2 concentrations of tert-OP, 4.5-6.5 and 10-30 µg/l, for 26 days in freshwater. Plasma VTG was induced in all treatments. A measure of smolting (gill Na+, K-ATPase activity, and osmoregulatory ability) was compromised at the higher tert-OP concentration. Downstream migratory behaviour in a stream raceway was observed in both control and treatment groups, but the fish that had been treated at the higher tert-OP concentration migrated at reduced frequency.	Bangsgaard *et al*. 2006
Adult male guppies (*Poecilia reticulata*) were exposed to tert-OP at a nominal 150 µg/l for 4 weeks, followed by another 10 d in clean water, after which their mating behaviour was recorded in the presence of non-receptive females. There was a significant reduction (~50%) in mating behaviour (duration and number of sigmoid displays) by comparison with controls.	Bayley *et al*. 1999
Juvenile brown trout (*Salmo trutta*) were exposed to tert-OP at 0.5-14.8 µg/l for 7-12 d. Plasma VTG was induced by a factor of >100 at 14.8 µg/l, giving a NOEC of 7.8 µg/l and an EC50 of 7 µg/l.	Bjerregaard *et al*. 2008
Male fathead minnow (*Pimephales promelas*) were exposed to tert-OP at nominal concentrations between 2.25 and 45 µg/l for 2 weeks and plasma VTG induction measured. The EC50 for induction was 48.2 µg/l.	Brian *et al*. 2005
Eyed embryos of rainbow trout (*Oncorhynchus mykiss*) were injected with tert-OP at 0.01, 0.1 and 1.0 mg/kg and studied for 6 months. No significant effects were recorded, including on sexual development.	Carlson *et al*. 2000
Adult male medaka (*Oryzias latipes*) were exposed to tert-OP by feeding contaminated diet for 7 days. The tert-OP concentrations ranged between 0.02	Chikae *et al*.

and 40 mg/g diet. The EC50 for plasma VTG induction was 2600 µg/g diet, with a 100,000-fold increase in VTG at the top concentration.	2003
Adult male cichlids *Cichlasoma dimerus* were exposed to tert-OP by injection (10 and 50 µg/g body wt.). Zona pellucida protein (ZPP) was measured in liver at 1-72 h and was significantly induced at 12 h after injection. Blood plasma and skin mucous also showed induction of both ZPP and VTG. Histological examination of testis showed abnormal amounts of sperm and immature germ cells, indicative of reproductive impairment, and liver histology showed elevated cellular activity consistent with ZPP and VTG induction.	Genovese *et al*. 2011
Medaka (*Oryzias latipes*) at a variety of times post-hatch were exposed to nominal concentrations of tert-OP at 100-300 µg/l for up to 3 months.100 µg/l induced testis-ova (in up to 20% of fish) in all groups exposed continuously for 3 months, although the effect was only statistically significant when the exposure began at 3 d post-hatch. Males in all treatments also showed abnormal spermatogonial development. There were no significant effects on sex ratios.	Gray *et al*. 1999a
Male and female medaka (*Oryzias latipes*) were exposed to nominal concentrations of tert-OP at concentrations between 10 and 100 µg/l from 1 d post-hatch to 6 months post-hatch. Males exposed to 25-50 µg/l (NOEC = 10 µg/l) showed reduction in courtship behaviour and ~50-80% reduction in overall reproductive success when mated with unexposed females. Fertilization success was also slightly but significantly reduced (NOEC <10 µg/l) and the % of developmental problems in offspring approximately doubled (NOEC <10 µg/l), either when just males were exposed, or when both males and females were exposed.	Gray *et al* 1999b
Male carp (*Cyprinus carpio*) exposed for 14 and 28 d to tert-OP demonstrated elevated plasma VTG at 4 µg/l (NOEC < 4 µg/l). When fish were transferred to clean water, VTG returned to control levels within 28 d.	Huang & Wang, 2001
Female eelpout (*Zoarces viviparus*) were exposed during early pregnancy to tert-OP at a nominal concentration of 100 µg/l. Plasma VTG was significantly elevated. 32% of embryos from treated mothers showed abnormal gonad development, including feminisation of males (no seminiferous tubules or sperm ducts).	Jespersen *et al*. 2010
Adult male rainbow trout (*Oncorhynchus mykiss*) were exposed to a measured tert-OP concentration of 38.5 µg/l for 21 d. This caused an increase in plasma VTG, and a decrease in testicular growth and spermatogenesis. Further exposures to a range of measured tert-OP concentrations (0.3-43.9 µg/l) gave a 21 d NOEC for VTG induction of 1.6 µg/l (LOEC = 4.8 µg/l) but no effects on gonadal size.	Jobling *et al*. 1996
Adult sheepshead minnow (*Cyprinodon variegatus*) were exposed for 24 d to measured levels of tert-OP at 11.5, 33.6 and 68.1 µg/l. All concentrations caused an elevation in plasma VTG (NOEC = < 11.5 µg/l), and there were abnormalities in testicular histology at 33.6 µg/l (NOEC = 11.5 µg/l).	Karels *et al*. 2003
Mature male and female guppies (*Poecilia reticulata*) were exposed to measured tert-OP at 26 µg/l, males for 28 d and females for 26-36 d until they gave birth. Offspring were raised in clean water to 70 days post-hatch. The main effect was a reduced number of spermatogenic cysts in the adult male testes, and no clear effects were seen in offspring.	Kinnberg *et al*. 2003
Mature male guppies (*Poecilia reticulata*) were exposed to measured tert-OP at 100-900 µg/l for 30-60 d. The only clear effects were seen at 900 µg/l, but many of these fish had died by 30 d. Testis histology in these fish showed increased spermatozeugmata and reduced numbers of spermatogenic cysts.	Kinnberg & Toft 2003
Juvenile rainbow trout (*Oncorhynchus mykiss*) were given intraperitoneal	Knudsen *et al*.

injections of tert-OP (5 or 50 mg/kg body wt.) and both doses produced a 2-fold upregulation of the hepatic estrogen receptor (ER) and a 3 to 5-fold upregulation of hepatic zona radiata protein (ZRP) (NOEC < 5 mg/kg). Combinations of tert-OP with either butylbenzylphthalate or estradiol did not produce more than additive effects on ER or ZRP induction.	1998
Adult male flounder (*Platichthys flesus*) were given a single oral dose of tert-OP (50 mg/kg body wt.) and plasma VTG induction studied. VTG was first detected at 48 h after dosing and continued increasing until the end of the experiment 9 days after dosing, when its concentration in plasma had reached 1.4 mg/ml. This was accompanied by a maximum concentration of tert-OP in liver of 67 µg/g which was observed 12 h after dosing.	Madsen *et al*. 2006
Adult male flounder (*Platichthys flesus*) were orally dosed on a semi-continuous basis for 11 d with tert-OP at 10, 50 or 100 mg/kg body wt. and VTG induction was measured in blood plasma. The maximum VTG measured was 10 mg/ml which occurred on day 10 in the fish dosed with 50 mg/kg (VTG induction NOEC < 10 mg/kg b.w.). Levels of tert-OP in liver were positively correlated with the VTG response, with the maximum concentration of hepatic tert-OP being approx. 50 µg/g.	Madsen *et al*. 2002
Adult male flounder (*Platichthys flesus*) were orally dosed on a semi-continuous basis for 10 d with tert-OP at 10, 50 or 100 mg/kg body wt. Plasma VTG was observed in all doses to a maximum of 10 mg/ml at the 50 mg/kg dose after 10 d. In another dosing experiment at lower concentrations, the 10 d VTG induction NOEC was established as 1 mg/kg b.w. VTG induction was positively correlated with tert-OP accumulation in the tissues, and the hepatic tert-OP concentration equivalent to the 10 d NOEC was approx. 0.02 µg/g.	Madsen *et al*. 2003
Immature male summer flounder (*Paralichthys dentatus*) were exposed by intra-sinus injection twice with tert-OP at 2, 20 and 200 mg/kg, and blood and tissues collected 4-8 weeks after first injection. Observed changes included lower gonadosomatic index and initially increased plasma estradiol (at 2 mg/kg) and decreased testosterone (at 200 mg/kg), but no effect on VTG production at any concentration.	Mills *et al*. 2001
Adult male zebrafish (*Danio rerio*) were exposed to tert-OP in water for 15 d. Observed effects included proliferation of liver peroxisomes and elevation of plasma VTG. There was also a correlation between VTG induction and an indicator of liver peroxisomal beta oxidation, acyl CoA oxidase activity.	Ortiz-Zarragoitia & Cajaraville 2005 [paper not seen]
Male killifish (*Fundulus heteroclitus*) were exposed to tert-OP by intraperitoneal injection (0, 10, 50, 100 and 150 mg/kg) and assessed for changes in gonadal histology and VTG induction. VTG was induced in a dose-dependent manner (NOEC = 50 mg/kg) and was a more sensitive biomarker than changes in testes (decreased GSI) which seemed to vary seasonally.	Pait & Nelson 2003)
Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to tert-octylphenol (branched) and n-octylphenol (linear) by either intraperitoneal injection (50 mg/kg) or water exposure (41 µg/l measured). I.P. injection of tert-OP induced plasma VTG after 12 d, but the linear form (n-OP) was inactive. Water exposure for 9 d also only gave a VTG response to the branched isomer.	Pedersen *et al*. 1999
Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to dietary 4 tert-OP administered semi-continuously for 11 d to doses ranging from 0.4 to 50 mg/kg/2 d. The NOEC for VTG induction in males (at least 1000-fold at the LOEC) was 20-30 mg/kg/2 d and the ED50 was 35 mg/kg/2 d. There was a positive correlation between the concentration of hepatic tert-OP and plasma VTG.	Pedersen *et al*. 2003

Pregnant eelpout (*Zoarces viviparus*) were exposed to tert-OP at measured concentrations of 14 or 65 µg/l for 35 d. Both concentrations induced plasma VTG in the mothers to maximum levels of >35 mg/ml, and the mass of the ovarian sac approximately doubled after 35 d at 65 µg/l. Embryonic weight was reduced by up to approx. 40% in both treatment groups and VTG was induced in embryos whose mothers were exposed to 65 µg/l. At the higher concentration, about 46% of embryos had normal ovaries, but only 22% had normal presumptive male gonads, and the remaining 32% had abnormal male gonads with an endo-ovarian cavity similar to that seen in normal females.	Rasmussen *et al*. 2002
Male eelpout (*Zoarces viviparus*) were exposed to tert-OP at a dose of 100 µg/g for 10 d. This caused an increase in plasma VTG and spermatocrit and a decrease in gonadosomatic index (GSI) and milt volume. The normal lobular appearance of the testis was disrupted, including the Sertoli cells, and the composition of seminal fluid was altered.	Rasmussen & Korsgaard 2004 [paper not seen]
Adults and secondary hermaphrodites of the cyprinodont fish *Kryptolebias marmoratus* were exposed to tert-OP at 300 µg/l from fertilisation to just post-hatch. The main observation was that tert-OP increased expression of the choriogenin gene Km-ChgH by a factor of approximately 3 in the hermaphrodites alone.	Rhee *et al*. 2009
Sand goby (*Pomatoschistus minutus*) were exposed to tert-OP for up to 6 months. After 28 d, immature males exposed to tert-OP at 31 or 101 µg/l showed elevated VTG mRNA expression, and plasma alkali-labile phosphate was elevated in both sexes. The 8 week LC50 for chronic effects was 29 µg/l, and both sexes demonstrated concentration- and duration-dependent increases in VTG mRNA expression. Exposure to tert-OP at 28 µg/l for 6 months inhibited development of male nuptial colouration and sperm duct glands.	Robinson *et al*. 2004
Juvenile rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) were exposed for 21 d to measured tert-OP concentrations of 1-100 µg/l. The NOEC for induced plasma VTG was 10 µg/l in both species.	Routledge *et al*. 1998
Adult male guppies (*Poecilia reticulata*) were exposed to tert-OP for up to 60 d at nominal concentrations of 100, 300 or 900 µg/l, and measured values varied by no more than 14% from nominal. There was substantial mortality in the top concentration, but the 2 lower concentrations produced an increased sperm count and reduced colouration index (secondary sexual characteristic). There were no clear effects on male reproductive capability or gonopodial length, but the gonadosomatic index (GSI) was reduced at the top and bottom concentrations.	Toft & Baatrup 2001
Newborn guppies (*Poecilia reticulata*) were exposed for 90 d to tert-OP at measured concentrations of 1.1, 11.7, 149 and 200 µg/l. This had no effect on sex ratio, but 149 µg/l altered male sexual behaviour and both sperm count and gonopodium length increased. On the other hand, at 149 µg/l the ovarian weight of females decreased. The NOEC for adverse effects was 11.7 µg/l.	Toft & Baatrup 2003
Adult male and female zebrafish (*Danio rerio*) were exposed to nominal concentrations of tert-OP at 12.5, 25, 50 and 100 µg/l for 3 weeks. Measured concentrations ranged from 75% to 56% of nominal. The GSI of non-spawning females was reduced at 25 µg/l and above (NOEC = 12.5 µg/l), but no other significant changes were observed.	Van den Belt *et al*. 2001
Male zebrafish (*Danio rerio*) and juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to nominal concentrations of 12.5-100 µg/l tert-OP under semi-static conditions for 3 weeks. As determined by VTG induction, tert-OP was not estrogenic in zebrafish, but VTG was induced in trout at 30 µg/l.	Van den Belt *et al*. 2003
Adult male and female cichlids (*Cichlasoma dimerus*) were exposed for 60 d to	Vázquez *et al*.

nominal semi-static tert-OP concentrations of 30, 150 and 300 µg/l. Measured concentrations dropped to 13% of their original value in the middle concentration over 72 h. VTG was detected in blood plasma and surface mucous of treated males at all concentrations (NOEC < 30 µg/l). Progressive effects on testicular histology (increased interstitial fibrosis; disarranged lobular structure; decrease or absence of spermatogonial cysts; abnormal spermatogenesis) were seen at 150 and/or 300 µg/l (NOEC = 30 µg/l). There were no major changes in ovarian histology.	2009
Juvenile male summer flounder (*Paralichthys dentatus*) were exposed to tert-OP (100-200 mg/kg) by 2 injections into the dorsal sinus, and fish were examined at 4, 6 and 8 weeks after the first injection. The main effect (at 200 mg/kg) was a reduction in testicular weight, sperm ducts full of sperm, numerous spermatogonia, and no developing sperm cycts. The liver was observed to produce a VTG-like substance which accumulated in liver, testis and kidney, causing pathology. Testicular maturation was not inhibited at any dose.	Zaroogian *et al*. 2001
Male crucian carp (*Carassius carassius*) were exposed for 14 d to tert-OP at nominal concentrations between 5 and 500 µg/l. Measured concentrations were consistent with the nominal ones, although these data were not presented. VTG was induced significantly by a factor of at least 400 at 20 µg/l and above (NOEC = 10 µg/l).	Zhang *et al*. 2010
<u>Studies in wildlife species : Amphibians</u>	
Clawed frog (*Xenopus laevis*) embryos at early gastrula stage (Gosner stage 10.5) were exposed to tert-OP at concentrations between 2 and 1032 µg/l for about 48 h until they reached stage 37. The high concentration (1032 µg/l) caused malformed cement glands, dorsal flexure, poorly developed somites, swollen guts or sloughing epidermal cells, but these were associated with very high mortalities. A lower concentration (103 µg/l) caused growth retardation and some body deformity (NOEC = 20.6 µg/l), but it is unclear whether these effects represented endocrine disruption.	Bevan *et al*. 2003
Newly-hatched larvae of the leopard frog (*Rana pipiens*) were exposed to nominal tert-OP concentrations of 0.2 and 200 µg/l for 10 d. Altered gene expression was observed in the hypothalamus at the higher concentration, but there were no effects on development or body weight. Relevance for endocrine disruption uncertain.	Crump *et al*. 2002
Tadpoles of wood frogs (*Rana sylvatica*) and northern leopard frogs (*Rana pipiens*) were exposed for 2 weeks to 8 nominal concentrations of tert-OP between 51 and 2063 µg/l. 14 d LC50 values ranged between 153 and 578 µg/l, depending on species and developmental stage. Some exposures also retarded body weight, but it is unclear whether this effect was related to endocrine disruption given the high exposure concentrations.	Hogan *et al*. 2006 [paper not seen]
Sexual differentiation of clawed frog (*Xenopus laevis*) larvae (2-3 d posthatch) was studied at 2 nominal concentrations of tert-OP (2.1 and 21 µg/l). Both concentrations produced an elevated number of female phenotypes compared to controls after 84 d exposure.	Kloas *et al*. 1999
Male and female tadpoles of the bullfrog *Rana catesbeiana* were exposed to tert-OP at 0.206, 2.06 or 20.63 µg/l for 24 h before and after the critical stages of sexual differentiation (Gosner stages 32-36). Both males and females exposed to the lowest dose and above (NOEC < 0.206 µg/l) underwent early gonadal	Mayer *et al*. 2003

differentiation, although males were particularly affected at stages 33 and 34. Furthermore, all tert-OP treatments abolished the sexually dimorphic expression of the orphan nuclear receptor steroidogenic factor-1 (SF-1) which normally occurs during sexual differentiation. SF-1 controls the expression of many steroidogenic enzymes, and these results suggest that low concentration of tert-OP may therefore be able to affect reproductive function in frogs. Sex ratios were, however, unaffected.	
Eggs of streamside salamander (*Ambystoma barbouri*) were exposed to tert-OP at measured concentrations of 5, 50 and 500 µg/l for 35 d. The NOEC for effects on time to hatch, larval survival and snout-vent length was 50 µg/l.	Rohr *et al*. 2003
Newly-hatched larvae and adult males of the snapping turtle (*Chelydra serpentina*) were exposed to a nominal tert-OP concentration of 10 µg/l for 17 and 35 d. Altered amyloid protein gene expression was observed in the hypothalamus, but there were no effects on growth or feeding rate. Relevance for endocrine disruption uncertain.	Trudeau *et al*. 2002
Adult male clawed frogs (*Xenopus laevis*) were exposed to intraperitoneally injected tert-OP at 100 mg/kg/week but it was unable to induce VTG in liver, although a VTG response was caused by 17-beta estradiol. However, there was a reduction in epithelium cell height and gland area in the nuptial glands.	Van Wyk *et al*. 2003
Studies in wildlife species: Birds	
Zebra finches (*Taeniopygia guttata*) were orally dosed with tert-OP during their linear growth phase (days 5-11) at 2 and 20 mg/kg body wt. Unlike treatment with estradiol, this caused no impacts on reproductive success in the resulting adults.	Millam *et al*. 2001
Zebra finches (*Taeniopygia guttata*) were orally dosed with tert-OP during their linear growth phase (days 5-11) at 206 mg/kg body wt. On day 12, oviducts were removed and weighed in an analogous way to the methods used in the rat uterotrophic assay. This treatment caused a 2-fold increase in oviduct weight, indicating estrogenic activity of tert-OP in this species.	Millam *et al*. 2002
Zebra finch chicks (*Taeniopygia guttata*) were orally dosed with tert-OP at 20.6 and 206 mg/kg body wt. during days 5-11 post-hatch. Unlike treatment with estradiol, this caused no increases in song control nuclei in the resulting adult females.	Quaglino *et al*. 2002
Studies in mammalian species	
Tert-OP (0, 10, 50, and 250 mg /kg/day) was administered orally to 5 week-old male SD rats (n=10) for three weeks. Effects on Sulphated Glycoprotein-2 (SGP-2), a biomarker for spermatogenesis, were determined in Sertoli cells of rat testes. Testicular expression of SGP-2 mRNA was decreased ($p<0.05$) in testes at all doses (15-40 % of the SGP-2 mRNA expression compared to controls).	Yon *et al* (2007)
Tert-OP (0, 10, 50, and 250 mg /kg/day) was orally administered to 5 week-old male SD rats (n=10) for three weeks. Effects on 3 ß-hydroxysteroid dehydrogenase/lyase (3ß-HSD), a key enzyme in steroidogenesis and a molecular marker for androgen biosynthesis was determined in Leydig cells. 3*ß*-HSD mRNA was decreased ($p<0.05$) in testes at all doses (up to 27 % of the control level) indicating that tert-OP may influence androgen biosynthesis in rat testes through an abnormal change in 3*ß*-HSD mRNA expression.	Kim *et al* (2007)
Male Fischer rats (4 weeks old) were administered tert-OP by s.c. at 0, 20, 40 or	Kim S.-K. *et al*

80 mg/kg bw/d, 3 times/week for one month (n=5). Effects on male reproductive organs were determined. Terminal body weights were significantly reduced in all tert-OP treated groups compared to controls. Reductions in the size and weight of the testis, epididymis, and seminal vesicle were observed in all the treated groups. Serum testosterone concentration was decreased while LH was increased. Seminiferous tubules were reduced in size and showed no mature spermatozoa or late-stage developing spermatids. In addition, testicular germ cells undergoing apoptosis were increased in all the treated groups. The expression of bcl-xL mRNA was significantly decreased in the OP treated groups, whereas the expressions of bcl-2 and bax mRNA were not significantly changed.	(2004)
Pregnant Wistar rats were administered tert-OP by gavage at 0, 15.6, 31.3, 62.5, 125, 250 (n=6), and 500 (n=7) mg/kg/day on pregnancy days 0 through day 8. Dams were terminated on day 20 of pregnancy. At 500 mg /kg/day all rats died and 2/6 rats treated with 250 mg /kg/day died. Some reductions in body weight gains occurred at doses > 31.5 mg /kg/day. There were no significant differences in the pregnancy rate between treated animals and the control group. The numbers of corpora lutea, implantation sites and pre-implantation loss per litter in the treated animals were not significantly different from the control. A significant decrease in the numbers of live fetuses per litter was observed at 31.5 and 125 mg/kg, and a significant increase in the incidence of post-implantation loss at dosages of $\geq$ 31.3 mg /kg/day. The sex ratio of live fetuses was comparable across all groups. Body weights of male and female fetuses was comparable across groups. No significant increases in the incidences of fetuses with external malformations were observed.	Harazono *et al*. (2001)
Dutch-Belted rabbit does (n=4-6 per group) were treated orally on alternate days between gestation days 15 and 30 with tert-OP at a dose of 150 mg /kg/day. Pups were killed at 24-26 weeks of age. Germ cell development and abdominal location of testes were investigated. One of four tert-OP pups was found to be unilaterally cryptorchid. Atypical germ cell resembling gonocytes and pre-spermatogonia were found in the undescended testis but not in testes of pups surgically treated on pnd 21 to remain cryptorchid.	Veeramachaneni (2006)
Pregnant SD rats were injected s.c. on days 13.5, 15.5, and 17.5 of pregnancy with tert-OP (0, 0.1, 1, 10, or 100 mg/kg). Fetuses were removed on gestation day 19.5, and effects on the prenatal testosterone surge were examined. In utero exposure to 4-tert-octylphenol had no effect on fetal body weight. Tert-OP at 100 mg/kg bw had a tendency to lower fetal testicular testosterone levels, but the effect was not statistically significant. When testes exposed in utero were taken into ex vivo tissue culture, testosterone secretion was similar to that of controls.	Haavisto *et al* (2003)
Pregnant Wistar rats (n=4/group) were given daily s.c. injections of tert-OP at 0, 100 or 250 mg /kg/day during pregnancy days 1-20. Effects on the male reproductive tract were examined. Final body weights of the male offspring from the 250 mg/kg bw/d group were significantly higher than those of the controls and the 100-mg group. There were no changes in testes weights between groups. Absolute right epididymis and prostate weights were increased in the 250-mg group; however, there were no differences between groups in relative organ weights. Histopathology revealed some changes in the epididymis of the 250 mg group. There were no changes between groups in organ weight of the caput epididymis and in epididymal sperm count. Sperm morphology revealed a slight increase in percentage of abnormal sperm in the high dose group. In a similar	Aydogan and Barlas (2006) Götekin and Barlas (2008)

study, effects on other endocrine organs were examined. No effects on the thyroid were reported.	
Pregnant NMRI mice were treated with s.c. injection of tert-OP at 0, 1 or 250 mg /kg/day from embryonic day 11.5 to ED 16.5. Effects on oocytes in newborn females were examined (n=8 pups). Ovarian morphology did not differ between groups. Tert-OP had no effect on the total number of oocytes or the percentage distribution of atretic, prefollicular, and follicular oocytes.	Sonne-Hansen *et al* (2003)
Pregnant SD rats (n=4/group) were given daily s.c. injections of tert-OP at 0, 10, 50 or 100 mg /kg/day during pregnancy days 0-5. Effects on growth and steroid synthesis in rat testis were examined. Tert-OP treated caused no histological changes in the testis and plasma testosterone, LH and FSH levels were not altered. Similarly, basal intratesticular testosterone concentrations as well as testicular testosterone concentration after hCG-stimulation did not differ from that of the controls. Basal progesterone secretion was unaffected but hCG-stimulated progesterone secretion was slightly elevated at 100 mg/kg bw/d. No changes in StAR or 3ß-HSD type I protein expression were detected.	Mikkilä *et al* (2006)
Male rat pups (Wistar) received s.c. injections of 2 mg tert-OP at serial doses from pnd 2-12, *i.e.* 11 doses altogether. This was equivalent to exposure to 150 mg /kg/day (Williams *et al*. 2001). Effects on the male reproductive system were determined. The morphology of the testis was unaffected but minor reductions in AQP-1 (water channel protein aquaporin-1) immunostaining were seen at at days 18 and/or 25. In animals that were followed through to day 35 days and/or adulthood, these changes were no longer obvious. Immunochemical expression of receptors (ERβ, ERα, AR and PR) were unchanged in seminal vesicles and seminal vesicle tissue composition was unaltered. Treatment with tert-OP had no effect on testis growth or on testosterone levels (except significantly elevated testosterone	Williams *et al* (2001a, b); Sharpe *et al* (2003)
Newborn female pups (n=6-8) were given daily s.c. injections of tert-OP at 0, 5 or 50 mg /kg/day from PNDs 1-10. Development of reproductive function was examined. No effect on body weight occurred. The age at vaginal opening was significantly earlier in rats treated 50 mg tert-OP/kg. At time of puberty, most of these animals did not show corpora lutea. Ovarian weights were lower and uterine weights higher in rats given 50 mg /kg/day compared to controls. Animals of the control group showed a prepubertal LH surge, whereas animals treated with 50 mg tert-OP/kg did not, and had consistently higher basal serum LH levels than the controls. After vaginal opening, all 50 mg tert-OP-treated animals (14/14) exhibited persistent oestrous, whereas all control animals had regular 4-5-d cycles. Histopathology of the ovaries showed reduced numbers of corpora lutea, and significantly higher numbers of preantral and atretic follicles.	Willoughby *et al* (2005)
Newborn female rat pups were injected with 100 mg/kg tert-OP s.c. within 24 h after birth. Administration was repeated every other day until pnd 15 (total of eight doses). Effects on the female reproductive tract were investigated. Before weaning, FSH and LH were lower than in the controls. Histologically, inhibition of uterine gland genesis was apparent. The day of vaginal opening was about 4 days earlier in tert-OP-treated animals than in controls. Persistent estrus was consistently observed in tert-OP-treated animals. Atrophic and polycystic ovaries without corpora lutea showed anovulation. In the endometrium, cell-proliferative activity and cell-death were increased and decreased, respectively, and expression of estrogen receptor alpha mRNA was apparent by in situ	Katsuda *et al* (2000b)

hybridization. Endometrial hyperplasias appeared at 8 weeks of age.	

Toxicokinetics in mammalian species

44. Results of individual studies are not shown. The following summary is taken from BAuA (2011).

"After oral application tert-OP is rapidly absorbed and quickly released into the blood. Within 10 min tert-OP is present in blood and Cmax is reached between 20 min (male Wistar rats) and 2 h (Sprague Dawley rats) after administration. Doses of 50 to 250 mg/kg bw resulted in Cmax of 40 to about 400 ng/ml blood. Repeated exposure to tert-OP of up to 125 mg/kg bw/d resulted in 2 to about 4-times higher blood concentrations in male and female animals than after single exposure. Indication for enterohepatic cycling was seen in some animals of one study, but not in two other studies. Cmax varies within the several tests depending on the applied dose and the strain used. Also the bioavailability varies between the strains. In male Wistar rats and female Da/Han rats oral bioavailabilties of about 10 % were determined. In male and female Sprague Dawley rats bioavailabilities were about 35 and 55 %, respectively.

45. Oral doses of 50 and 200 mg/kg bw/d resulted in low tert-OP concentrations in lung and tissue of male Wistar rats (7-9 ng/g tissue). tert-OP was detected at concentrations of 43- 87 ng/g tissue in muscle, kidney and liver and a concentration of 1285 ng/g in fat, but not in testes. In Sprague Dawley rats 4-tert-octylphenol could be detected in a dose-dependent manner at concentrations in the single-digit microgram range per gram tissue after administration of 25 to 125 mg/kg bw/d. Tissue concentrations were highest in liver and fat and also reaches reproductive organs such as uterus, ovaries, testes and the epididymis. After repeated doses no significant differences occurred between the tissue concentrations from single and repeated treatment indicating no bioaccumulation of tert-OP.

46. From experiments using rat liver perfusion or primary rat hepatocytes it can be concluded that tert-OP undergoes a rapid first pass metabolism by phase I and phase II enzymes in the liver. Detoxification pathways include hydroxylation, glucuronidation and sulphation. Enzymes involved in phase II metabolism include rat and human UGT2B1 and human SULT 1E1 and 2A1, as shown in *in vitro* experiments. In an *in vitro* test with untreated rat liver microsomes up to 94 % of tert-OP was metabolized within 15 min. Further studies showed that tert-OP may have a direct inhibitory effect on cytochrome P450 activities, and can decrease protein levels of testosterone hydroxylating CYP activities in the liver, when rats were fed 4-tert-octylphenol. In a liver perfusion assay 38% of the applied tert-OP dose was directly excreted into the bile of Sprague Dawley rats as glucuronide.

47. In Sprague Dawley rats some gender differences were observed in terms of a higher oral bioavailability and an increased terminal half life of tert-OP in females compared to males after oral application. This leads to a slower degradation *in vivo* and is in line with an *in vitro* investigation that showed a slower degradation of tert-OP by liver microsomes of female SD rats compared to males."

5.2. Analysis of Building Blocks Using 4-tert-Octylphenol Data

48. Each assay for which guidance is given in the draft GD has been evaluated using the relevant data for octylphenol inserted into the building block in the draft GD. The approach used is described in Section 3.2 (above). The wording in each series of tables is designed to mirror that used in the building blocks (BBs) in the draft GD. The results of the assays have been abbreviated here for clarity but more detail is given in Tables 5.1 and 5.2.

5.3. 4-Tert-Octylphenol Case Study Results: Validated *in vitro* Assays

5.3.1. ER Binding Assay (US EPA OPPTS 890.1250) (GD No. 150 Section C.2.1)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that tert-OP binds to ER, while existing *in vitro* data show a variety of responses including AR binding, ER agonism and AR antagonism, steroidogenesis disruption and possibly TH disruption. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.3.1.1: tert-OP data summary

ER Binding Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
In standard assays using rat uterine cytosol, tert-OP displaced E2 with IC50 of 2-12 μM and Ki 0.8-30 μM. (Laws *et al*, 2000, 2006). Similar results were obtained in non-standard assays using hER α and β and rat uterine cytosol (Strunck *et al*, 2000; Yoon	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data

ER Binding Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
et al, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010). Tert-OP was also positive for ER binding in assays using ER prepared from various wildlife species: fish (Andreassen & Korsgaard 2000; Denny *et al*. 2005; frog (Lutz & Kloas 1999)	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009)		

ER Binding Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005). PR binding (weak affinity) (Laws *et al* 2000)	some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c.caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	

Table 5.3.1.2: Conclusions about ER Binding Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
Tert-OP interacts with ER (from mammalian and non-mammalian species), combined with effects on AR. Some possible effect on S and T. Potential for adverse effects via multiple mechanisms	Perform assay ER STTA (level 2)	ER STTA: positive Agonism, EC50 approx 1 µM. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006).
	or assay from upper levels *e.g.* UT assay (level 3)	UT assay : positive ER agonism. Increase in uterine weight at oral doses of 100 mg/kg/day and above. Uterine weight increase after s.c. administration of 50 mg/kg/day and above. (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).
	or female PP assay (level 4)	Female PP assay : positive ER agonism. Age at VO was advanced at 200 mg/kg/day. No

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
		other parameters were determined (Laws *et al*, 2000).
	or ext-1 or 2-gen assays (level 5)	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.
	or partial/full fish life cycle tests (level 4/5).	A standard full life cycle test with medaka (Japanese Ministry of the Environment 2006) gave NOECs for induction of ♂ VTG of 4.3 µg/l, for induction of testis-ova of 9.9 µg/l. However, there were no effects on growth, GSI or time to hatching, and equivocal effects on hatchability at 82 µg/l. In a life cycle test with zebrafish, Segner *et al*. (2003) reported reduced fertilisation success with a 78 d EC50 of 28 µg/l. In another test with zebrafish, Wenzel *et al*. (2001) did not show effects on sex ratio, but reported reduced growth, fecundity and fertility with NOECs of 12 µg/l. In non-standard long-term tests, Gray *et al* (1999b) showed a significant reduction in sexual behaviour and overall reproductive success of medaka (NOEC = 10 µg/l) and a reduction in fertilisation success (NOEC <10 µg/l); and Robinson *et al*. 2004 showed that sand goby nuptial colouration and development of sperm duct glands was inhibited after 6 months at 28 µg/l; On the other hand, Toft & Baatrup (2001; 2003) failed to demonstrate altered sex ratios or reduced reproductive capability in guppies at concentrations up to 900 µg/l.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
		In summary, some fish life cycle tests showed testis-ova and adverse effects on overall reproductive success, the lowest NOEC for such adverse impacts being 9.9 µg/l.

Table 5.3.1.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for Tert-OP
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Guideline level 5 study on tert-OP provides an adequate assessment by a relevant route (oral exposure) (Tyl *et al*, 1999). However this study lacks some endocrine endpoints present in more recent study designs (TG 443). The lack of effect via the oral route is supported by other oral studies in intact mammals. Studies using s.c. administration indicate that effects could be observed via this route (that may deliver a larger internal dose) but data are contradictory.
If existing data are from level 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g.* FSDT) may be sufficient for this purpose.	The female PP assay alone (level 4) on tert-OP does not provide sufficient data for ED assessment. Few endpoints were determined (Laws *et al*, 2000). Adequate level 5 type studies are therefore required. Even if a full PP assay had been conducted it is likely that a level 5 study would be required so that a more comprehensive assessment could be made. Both Level 4 and 5 fish assays(Japanese Ministry of the Environment 2006; Segner *et al*., 2003; Wenzel *et al*. 2001; Gray *et al*., 1999a&b; Robinson *et al*., 2004; Toft & Baatrup, 2001& 2003; Knörr & Braunbeck 2002; Seki *et al*. 2003, and one non-standard Level 4 amphibian assay (Kloas *et al*., 1999) suggest that ER-binding leads to adverse effects on sexual development and/or reproduction, with similar aquatic NOECs. However, a non-standard bird reproduction assay detected no effects (Millam *et al*. 2001)
If existing data are from UT assay then level 4 assay will provide data on multiple modalities.	UT assay on tert-OP demonstrates ER agonism *in vivo* (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a). The level 4 PP assay adds to these

Other considerations (as given in BB)	Conclusions for Tert-OP
	observations
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Tert-OP appears to produce endocrine effects at a lower dose, when administered s.c rather than orally. This is consistent with rapid first pass metabolism via the oral route and the potential to achieve higher internal doses when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	Tert-OP is metabolised by hydroxylation, glucuronidation and sulphation. The glucuronide metabolite had no activity when tested in ER and AR transactivation assays for agonism and antagonism. This indicates that glucuronide conjugation is a detoxification reaction (Moffat et al, 2001).

49. Overall conclusions about ER Binding Assay, and existing data: Next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence that tert-OP can bind to ER and that binding produces an agonist response *in vitro.* Interaction with other endocrine systems was also shown *in vitro*. Positive results in mammalian *in vivo* screening assays (*e.g.* the UT assay) for ER agonism demonstrate that this response can also occur *in vivo*. In mammalian species, the available evidence from a level 5 assay indicates that this interaction does not result in effects on reproduction when administration is via the the oral route. Other routes, where a higher internal dose may be achieved, may give different results. Interestingly, the reverse is the case in fish and amphibians, where a variety of effects on sexual development and reproduction were caused by tert-OP.

5.3.2 AR Binding Assay (US EPA OPPTS 890.1150) (GD No. 150 Section C.2.2)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that tert-OP binds to AR, while existing *in vitro* data show a variety of responses including ER binding, ER agonism and AR antagonism, steroidogenesis disruption and possibly TH disruption. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.3.2.1: tert-OP data summary

AR Binding Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
In standard assays with hAR, tert-OP displaced R1881 with IC50 of approximately 2µM and Ki 25 µM. (Paris *et al* 2002; Li *et al* 2010).	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	

AR Binding Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). s.c. with 100 or 50 mg/kg/day caused changes such as	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009)		
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)		

AR Binding Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	

Table 5.3.2.2: Conclusions about AR Binding Assay, and existing data and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
Tert-OP interacts with AR combined with effects on ER. Some possible effect on S and T. Potential for adverse effects via multiple mechanisms	Perform assay (level 2) AR STTA	AR STTA: positive Antagonism, IC50 1-3 µM. (Krüger *et al* 2008; Paris *et al* 2002).
	or assay from upper levels *e.g.* H assay (level 3)	H assay : negative No agonistic or antagonistic effects were seen in the castrated rat assay at oral doses of 50 to 200 mg/kg/day (Yamasaki *et al*, 2003).
	or fish screen (AFSS) (level 3)	No data are available froman AFSS.
	or male PP assay (level 4)	Not available.
	or ext-1 or 2-gen assays (level 5)	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.
	or partial/full fish life cycle tests (level 4/5).	The available fish and amphibian partial/full lifecycle data (Japanese Ministry of the Environment 2006; Segner *et al*., 2003; Wenzel *et al*. 2001; Gray *et al*., 1999a&b; Robinson *et al*., 2004; Toft & Baatrup, 2001& 2003; Knörr & Braunbeck 2002;

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
		Seki *et al*. 2003; Kloas *et al*., 1999) show adverse effects on sexual development and reproduction which, taken together, are probably attributable to the estrogenic effects of tert-OP,and not its interactions with the androgen receptor.

Table 5.3.2.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for Tert-OP
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Guideline level 5 study on tert-OP provides an adequate assessment by a relevant route (oral exposure) (Tyl *et al*, 1999). However this study lacks some endocrine endpoints present in more recent study designs (TG 443). The lack of effect via the oral route is supported by other oral studies in intact mammals. Studies using s.c. administration indicate that effects could be observed via this route (that may deliver a larger internal dose) but data are contradictory.
If existing data are from level 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g*. FSDT) may be sufficient for this purpose.	No male PP assay is available and the female PP assay (level 4) on tert-OP does not provide sufficient data for ED assessment. Few endpoints were determined (Laws *et al*, 2000). Adequate level 5 type studies are therefore required. Even if full PP assays had been conducted it is likely that a level 5 study would be required so that a more comprehensive assessment could be made. Both Level 4 and 5 fish assays(Japanese Ministry of the Environment 2006; Segner *et al*., 2003; Wenzel *et al*. 2001; Gray *et al*., 1999a&b; Robinson *et al*., 2004; Toft & Baatrup, 2001& 2003; Knörr & Braunbeck 2002; Seki *et al*. 2003, and one non-standard Level 4 amphibian assay (Kloas *et al*., 1999) show adverse effects on sexual development and/or reproduction probably attributable to ER binding and not AR binding. However, a non-standard bird reproduction assay detected no effects (Millam *et al*. 2001)
If existing data are from H assay or AFSS then level 4 mammalian assay or fish screen (TG 229/230) will provide data on multiple modalities.	H assay on tert-OP is negative for agonism and antagonism (Yamasaki *et al*, 2003). Level 4 PP assay would provide more conclusive data especially given the conflicting results in repeat

Other considerations (as given in BB)	Conclusions for Tert-OP
	dosing studies. No data are available from an AFSS.
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Tert-OP appears to produce endocrine effects at a lower dose, when administered s.c rather than orally. This is consistent with rapid first pass metabolism via the oral route and the potential to achieve higher internal doses when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	Tert-OP is metabolised by hydroxylation, glucuronidation and sulphation. The glucuronide metabolite had no activity when tested in ER and AR transactivation assays for agonism and antagonism. This indicates that glucuronide conjugation is a detoxification reaction (Moffat et al, 2001).

50. Overall conclusions about AR Binding Assay and existing data: Next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence that tert-OP can bind to AR and that binding produces an antagonist response *in vitro.* Interaction with other endocrine systems was also shown *in vitro*. In the H assay (mammalian *in vivo* screening assay at level 3) tert-OP was negative indicating that the AR antagonism observed *in vitro* did not produce a similar response in vivo, under the conditions of the assay. In mammalian species, the available evidence from a level 5 assay indicates that this interaction does not result in effects on reproduction when administration is via the oral)route. Other routes, where a higher internal dose may be achieved, may give different results. Interestingly, the reverse is the case in fish and amphibians, where a variety of effects on sexual development and reproduction were caused by tert-OP. .

5.3.3 OECD TG 455: The Stably Transfected Human ERα Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA) (including Guidance for the Antagonism Assay) (GD No. 150 Section C.2.3)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that tert-OP shows an agonist response in ER transactivation assays, while existing *in vitro* data show a variety of responses including ER binding, AR binding and AR antagonism, steroidogenesis disruption and possibly TH disruption. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.3.3.1: Tert-OP data summary

Human ERα Transcriptional Activation Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Tert-OP increased reporter gene activity (agonist) with EC50 of 0.1 μM (OECD 2006a). Similar results were obtained in non-standard assays using hER (Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006 and in transactivation assays using ER derived from fish (Fu *et al*. 2007; Gale *et al*. 2004).	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal	

Human ERα Transcriptional Activation Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009)		
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)		

Table 5.3.3.2: Conclusions about Human ER-alpha Transcriptional Activation Assay, and existing data and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for Tert-OP
Tert-OP is an ER agonist, combined with effects on AR, S (and possibly T). Potential for adverse effects via multiple mechanisms.	Perform assay from upper levels *e.g.* UT assay (level 3)	UT assay : positive ER agonism. Increase in uterine weight at oral doses of 100 mg/kg/day and above. Uterine weight increase after s.c. administration of 50 mg/kg/day and above.

Conclusions for tert-OP	Next Step (as BB)	Assay result for Tert-OP
		(Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).
	or female PP assay (level 4)	Female PP assay : positive ER agonism. Age at VO was advanced at 200 mg/kg/day. No other parameters were determined (Laws *et al*, 2000).
	or ext-1 or 2-gen assays (level 5	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.
	or partial/full fish life cycle tests (level 4/5).	A standard full life cycle test with medaka (Japanese Ministry of the Environment 2006) gave NOECs for induction of ♂ VTG of 4.3 µg/l, for induction of testis-ova of 9.9 µg/l. However, there were no effects on growth, GSI or time to hatching, and equivocal effects on hatchability at 82 µg/l. In a life cycle test with zebrafish, Segner *et al*. (2003) reported reduced fertilisation success with a 78 d EC50 of 28 µg/l. In another test with zebrafish, Wenzel *et al*. (2001) did not show effects on sex ratio, but reported reduced growth, fecundity and fertility with NOECs of 12 µg/l. In non-standard long-term tests, Gray *et al* (1999b) showed a significant reduction in sexual behaviour and overall reproductive success of medaka (NOEC = 10 µg/l) and a reduction in fertilisation success (NOEC <10 µg/l); and Robinson *et al*. 2004 showed that sand goby nuptial colouration and development of sperm duct glands was inhibited

Conclusions for tert-OP	Next Step (as BB)	Assay result for Tert-OP
		after 6 months at 28 µg/l; On the other hand, Toft & Baatrup (2001; 2003) failed to demonstrate altered sex ratios or reduced reproductive capability in guppies at concentrations up to 900 µg/l. In summary, some fish life cycle tests showed testis-ova and adverse effects on overall reproductive success, the lowest NOEC for such adverse impacts being 9.9 µg/l.

Table 5.3.3.3: Other considerations and conclusions for tert-OP

Other considerations (as given in BB)	conclusions for Tert-OP
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Guideline level 5 study on tert-OP provides an adequate assessment by a relevant route (oral exposure) (Tyl *et al*, 1999. However this study lacks some endocrine endpoints present in more recent study designs (TG 443). The lack of effect via the oral route is supported by other oral studies in intact mammals. Studies using s.c. administration indicate that effects could be observed via this route (that may deliver a larger internal dose) but data are contradictory.
If existing data are from level 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g*. FSDT) may be sufficient for this purpose.	The female PP assay alone (level 4) on tert-OP does not provide sufficient data for ED assessment. Few endpoints were determined (Laws *et al*, 2000). Adequate level 5 type studies are therefore required. Even if a full PP assay had been conducted it is likely that a level 5 study would be required so that a more comprehensive assessment could be made. Both Level 4 and 5 fish assays(Japanese Ministry of the Environment 2006; Segner *et al*., 2003; Wenzel *et al*. 2001; Gray *et al*., 1999a&b; Robinson *et al*., 2004; Toft & Baatrup, 2001& 2003; Knörr & Braunbeck 2002; Seki *et al*. 2003, and one non-standard Level 4 amphibian assay (Kloas *et al*., 1999) suggest that ER-binding leads to adverse effects on sexual development and/or reproduction, with similar aquatic NOECs. However, a non-standard bird reproduction assay detected no effects (Millam *et al*. 2001)
If existing data are from UT assay then level 4 mammalian assay or fish screen (TG 229/230)	UT assay on tert-OP demonstrates ER agonism *in vivo* (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002;

Other considerations (as given in BB)	conclusions for Tert-OP
will provide data on multiple modalities.	Yamasaki *et al* 2002; Katsuda *et al* 2000a). The level 4 PP assay adds to these observations
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Tert-OP appears to produce endocrine effects at a lower dose, when administered s.c rather than orally. This is consistent with rapid first pass metabolism via the oral route and the potential to achieve higher internal doses when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	Tert-OP is metabolised by hydroxylation, glucuronidation and sulphation. The glucuronide metabolite had no activity when tested in ER and AR transactivation assays for agonism and antagonism. This indicates that glucuronide conjugation is a detoxification reaction (Moffat et al, 2001).

51. Overall conclusions about Human ER-alpha Transcriptional Activation Assay, and existing data: Next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence that the binding of tert-OP to ER results in gene activation and an agonist response *in vitro.* Interaction with other endocrine systems was also shown *in vitro*. Positive results in mammalian *in vivo* screening assays (*e.g.* the UT assay) for ER agonism demonstrate that this response can also occur *in vivo*. In mammalian species, the available evidence from a level 5 assay indicates that this interaction does not result in effects on reproduction when administration is via the oral route. Other routes, where a higher internal dose may be achieved, may give different results. Interestingly, the reverse is the case in fish and amphibians, where a variety of effects on sexual development and reproduction were caused by tert-OP.

5.3.4. OECD TG 456: H295R Steroidogenesis Assay (GD No. 150 Section C.2.4)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that tert-OP disrupts steroidogenesis, while existing *in vitro* data show a variety of responses including ER binding, AR binding, ER agonism and AR antagonism and possibly TH disruption. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.3.4.1: tert-OP data summary

H295R Steroidogenesis Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
Steroidogenesis disruption was	ER binding to mammalian and wildlife isoforms.	TG 407 - reduced SAT weights in rats (♂) at 450	No data

H295R Steroidogenesis Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
observed in a non-standard assay (rat Leydig cells). Exposure to up to to 2 μM increased testosterone levels (up to 2-fold) (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003).	(Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et*	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	

H295R Steroidogenesis Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	al. 1994)		
	AR antagonism: AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009)		
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)		

Table 5.3.4.2: Conclusions about Steroidogenesis Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
Tert-OP inhibits steroidogenesis,	Perform assay from upper levels	Female PP assay : positive

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
in combination with effects on ER, AR (and possibly T). Potential for adverse effects via multiple mechanisms	*e.g.* male or female pubertal assay (level 4)	ER agonism. Age at VO was advanced at 200 mg/kg/day. No other parameters were determined (Laws *et al*, 2000). Male PP assay: not available.
	or ext-1 or 2-gen assays (level 5)	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.
	or partial/full fish life cycle tests (level 4/5).	A standard full life cycle test with medaka (Japanese Ministry of the Environment 2006) gave NOECs for induction of ♂ VTG of 4.3 µg/l, for induction of testis-ova of 9.9 µg/l. However, there were no effects on growth, GSI or time to hatching, and equivocal effects on hatchability at 82 µg/l. In a life cycle test with zebrafish, Segner *et al*. (2003) reported reduced fertilisation success with a 78 d EC50 of 28 µg/l. In another test with zebrafish, Wenzel *et al*. (2001) did not show effects on sex ratio, but reported reduced growth, fecundity and fertility with NOECs of 12 µg/l. In non-standard long-term tests, Gray *et al* (1999b) showed a significant reduction in sexual behaviour and overall reproductive success of medaka (NOEC = 10 µg/l) and a reduction in fertilisation success (NOEC <10 µg/l); and Robinson *et al*. 2004 showed that sand goby nuptial colouration and development of sperm duct glands was inhibited after 6 months at 28 µg/l;

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
		On the other hand, Toft & Baatrup (2001; 2003) failed to demonstrate altered sex ratios or reduced reproductive capability in guppies at concentrations up to 900 µg/l. In summary, some fish life cycle tests showed testis-ova and adverse effects on overall reproductive success, the lowest NOEC for such adverse impacts being 9.9 µg/l.

Table 5.3.4.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	conclusions for tert-OP
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Guideline level 5 study on tert-OP provides an adequate assessment by a relevant route (oral exposure) (Tyl *et al*, 1999). However this study lacks some endocrine endpoints present in more recent study designs (TG 443). The lack of effect via the oral route is supported by other oral studies in intact mammals. Studies using s.c. administration indicate that effects could be observed via this route (that may deliver a larger internal dose) but data are contradictory.
If existing data are from level 3 or 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment, although some Level 4 assays (*e.g*. FSDT) may be sufficient for this purpose.	No male PP assay is available and the female PP assay (level 4) on tert-OP does not provide sufficient data for ED assessment. Few endpoints were determined (Laws *et al*, 2000). Adequate level 5 type studies are therefore required. Even if full PP assays had been conducted it is likely that a level 5 study would be required so that a more comprehensive assessment could be made. Both Level 4 and 5 fish assays(Japanese Ministry of the Environment 2006; Segner *et al*., 2003; Wenzel *et al*. 2001; Gray *et al*., 1999a&b; Robinson *et al*., 2004; Toft & Baatrup, 2001& 2003; Knörr & Braunbeck 2002; Seki *et al*. 2003, and one non-standard Level 4 amphibian assay (Kloas *et al*., 1999) show adverse effects on sexual development and/or reproduction, with similar aquatic NOECs. Given strong induction of VTG in males, it is likely that these effects are caused primarily by agonistic interactions with the ER,

Other considerations (as given in BB)	conclusions for tert-OP
	although simultaneous effects on steroidogensis cannot be ruled out. However, a non-standard bird reproduction assay detected no effects (Millam *et al*. 2001)
Compare steroidogenesis assay results with other *in vitro* results to help discern mechanism.	The results described in rat Leydig cells are reported to be independent of ER. In an H295R assay, cortisol was reduced and some some enzymes inhibited, indicating possible effects on these pathways (Nakajiin *et al* 2001).
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Tert-OP appears to produce endocrine effects at a lower dose, when administered s.c rather than orally. This is consistent with rapid first pass metabolism via the oral route and the potential to achieve higher internal doses when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	Tert-OP is metabolised by hydroxylation, glucuronidation and sulphation. The glucuronide metabolite had no activity when tested in ER and AR transactivation assays for agonism and antagonism. This indicates that glucuronide conjugation is a detoxification reaction (Moffat et al, 2001).

52. Overall conclusions about Steroidogenesis Assay and existing data,: Next steps and other considerations provide a logical course of action to follow if the assay had been conducted according to OECD TG 456. As this was not the case, the first course of action should be to repeat the study following the guideline. The positive result using a rat Leydig cell steroidogenesis assay indicates that the result is likely to be positive. The combined dataset provides evidence that tert-OP can interact with a number of endocrine systems. In mammalian species, the available evidence from a level 5 assay indicates that this interaction does not result in effects on reproduction, when administration is via the oral route. Other routes, where a higher internal dose may be achieved, may give different results. Interestingly, the reverse is the case in fish and amphibians, where a variety of effects on sexual development and reproduction are caused by tert-OP.

5.3.5 Aromatase Assay (US EPA OPPTS 890.1200) (GD No. 150 Section C.2.5)

No data available

5.4 4-Tert-Octylphenol Case Study Results: Validated Wildlife *in vivo* Assays

5.4.1 OECD TG 229: Fish Short Term Reproduction Assay (FSTRA) (GD No. 150 Section C.3.1)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows depressed fecundity and ♂ secondary sexual characteristics, and elevated ♂ VTG, while existing *in vitro* data show a variety of responses consistent with ER interactions, and existing *in vivo* data show a variety of endpoints consistent with ER agonism.

Table 5.4.1.1: Tert-OP data summary

Fish Short Term Reproduction Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
♂ VTG↑, ♂ secondary sexual characteristics ↓, fecundity and fertility ↓. LOECs for effects on VTG in the range 0.6-37 µg/l, and for effects on secondary sexual characteristics in the range 0.6-113 µg/l. LOECs for reduced fecundity and fertility are 113 µg/l. (Biever *et al*. 2007). A non-standard assay in which females were not exposed to tert-OP gave a NOEC for ↑♂ VTG of 20 µg/l and for ↓ fecundity of <20 µg/l (Gronen *et al*., 1999)	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	TG 230: ♂ VTG↑ (NOEC = 27.8 µg/l) (Japanese Ministry of the Environment 2006). In a non-standard assay, hepatic ♂ VTG ↑ (NOEC = 27.8 µg tert-OP/l) (Nozaka *et al*. 2004). These results support those obtained with TG 229.
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009). TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	Non-standard short-term studies with fish show ♂ and ♀ VTG ↑ (or ♂ VTG mRNA transcripts ↑; and/or ♂ ZPP/ZRP ↑) in a large range of species after either tert-OP injection, feeding or ambient exposure. Ambient water NOECs for these effects ranged from 1.6 to 10 µg/l. (Andreassen & Korsgaard 2000; Andreassen *et al*. 2005; Bangsgaard *et al*. 2006; Bjerregaard *et al*. 2008; Brian *et al*. 2005; Chikae *et al*. 2003; Genovese *et al*. 2011; Huang & Wang, 2001; Jespersen *et al*. 2010; Jobling *et al*. 1996; Karels *et al*.
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	

Fish Short Term Reproduction Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	2003; Knudsen *et al*. 1998; Madsen *et al*. 2002, 2003, 2006; Ortiz-Zarragoitia & Cajaraville 2005; Pait & Nelson 2003; Pedersen *et al*. 1999, 2003; Rasmussen & Korsgaard 2004; Routledge *et al*. 1998; Van den Belt *et al*. 2003; Zhang *et al*. 2010). Some of these studies also reported reductions in milt volume, GSI, smolting ability, growth and mating behaviour, or abnormalities in testicular histology and spermatocrit. The results of these studies largely support the results obtained with TG 229.
		Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as	
	AR antagonism: AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).		
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).		
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth Ghisari *et al* 2009).		
	Some effects on hormone secretion from isolated rat ovarian follicles.		A non-standard short-term assay with adult male clawed frogs

Fish Short Term Reproduction Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	(Myllymaki *et al*, 2005).	premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	failed to induce VTG after i.p. injection (100 mg/kg/week), although this response was triggered by E2 (Van Wyk *et al*. 2003). ♀ and ♂ bullfrog tadpoles exposed short-term in a non-standard test to tert-OP underwent early gonadal differentiation (NOEC < 0.206 µg/l) (Mayer *et al*. 2003).
	PR binding (weak affinity) (Laws *et al* 2000)		
			Short term exposures of zebra finch chicks to orally dosed tert-OP (206 mg/kg body wt.) caused increases in oviduct weight (Millam *et al*. 2002).

Table 5.4.1.2: Conclusions about Fish Short Term Reproduction Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
Strong evidence for *in vivo* endocrine activity with potential adverse effects (reproductive toxicity) in fish	Consider performing a fish lifecycle test, especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	A standard full life cycle test with medaka (Japanese Ministry of the Environment 2006) gave NOECs for induction of ♂ VTG of 4.3 µg/l, for induction of testis-ova of 9.9 µg/l. However, there were no effects on growth, GSI or time to hatching, and equivocal effects on hatchability at 82 µg/l. In a life cycle test with zebrafish, Segner *et al*. (2003) reported reduced fertilisation success with a 78 d EC50 of 28 µg/l. In another test with zebrafish, Wenzel *et al*. (2001) did not show effects on sex

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
		ratio, but reported reduced growth, fecundity and fertility with NOECs of 12 µg/l. In non-standard long-term tests, Gray *et al* (1999b) showed a significant reduction in sexual behaviour and overall reproductive success of medaka (NOEC = 10 µg/l) and a reduction in fertilisation success (NOEC <10 µg/l); and Robinson *et al*. 2004 showed that sand goby nuptial colouration and development of sperm duct glands was inhibited after 6 months at 28 µg/l; On the other hand, Toft & Baatrup (2001; 2003) failed to demonstrate altered sex ratios or reduced reproductive capability in guppies at concentrations up to 900 µg/l. In summary, some fish life cycle tests showed testis-ova and adverse effects on overall reproductive success, the lowest NOEC for such adverse impacts being 9.9 µg/l. The various NOECs were in the same range, or higher, than LOECs and NOECs observed in standard and non-standard versions of TG 229, and confirm that this screening assay gave an alert for effects on reproduction.

Table 5.4.1.3: Other considerations and conclusions for octylphenol. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for tert-OP
An alternative approach would be to deploy the fish sexual development test, especially if sexual development is expected to give a response at lower concentrations than reproduction.	In a standard FSDT (OECD, 2011), zebrafish gave NOECs of 9.5-17.6 µg/l for ♂VTG induction and 5.7-<13.8 µg/l for ♀-biased sex ratio and Japanese medaka gave

Other considerations (as given in BB)	Conclusions for tert-OP
	NOECs of 6.2 -31.7 µg/l for ♂and ♀ VTG induction and <11.2-23.5 µg/l for ♀-biased sex ratio. Three-spined sticklebacks were a little less sensitive with NOECs of 22.5->41.9 µg/l for ♂VTG induction and >41.9-66.0 µg/l for undifferentiated biased sex ratio. A test with medaka (Knörr & Braunbeck 2002) gave a NOEC of 20 µg/l for ♀-biased sex ratio. A second test with medaka (Seki *et al*. 2003) gave a NOEC for ♀-biased sex ratio of 23.7 µg/l. In a non-standard test similar to the FSDT, Gray *et al*. (1999a) demonstrated a NOEC of <100 µg/l for testis-ova induction in medaka. However, Toft & Baatrup (2003) reported no effect on sex ratio in guppies at concentrations up to 200 µg/l, but the NOEC for adverse effects on sperm count and gonopodial length was 11.7 µg/l. In summary, ♀-biased sex ratio in 2 species (zebrafish and medaka) occurred with NOECs in the range 5.7-23.7 µg/l. These NOECs were in a similar range to NOECs for adverse effects derived from full lifecycle tests, and equal to or higher than NOECs derived from TG 229. On the other hand, a third species (guppy) did not show biased sex ratio at concentrations up to 200 µg/l.
The decision about whether to conduct a FLCTT or MMGT may be driven primarily by the bioaccumulative properties of the chemical – a one generation test (*e.g.* F0 eggs to F1 fry) will generally be sufficient if the chemical is not expected to be transferred to the fry via the eggs.	No MMGT data are available. However, tert-OP is not strongly bioaccumulative, and may therefore not be significantly less potent in the FLCTT compared with the MMGT.

53. Overall conclusions about Fish Short Term Reproduction Assay and existing data: In the light of estrogenic effects on ♂ VTG and ♀ fecundity demonstrated in TG 229, the BB conclusion that adverse effects on reproduction might occur, and the advice to conduct a fish full life cycle or fish sexual development test, were vindicated because these tests (in some species) revealed adverse effects on sex ratio and a variety of reproductive endpoints including overall reproductive success. The NOECs for adverse effects in both the partial and full fish lifecycle tests were similar to, or higher, than NOECs reported for TG 229. The limited data from amphibians did not strongly predict the estrogenic effects in fish, but limited data from birds and abundant data from mammals were both predictive of such effects.

5.4.2 OECD TG 230: 21 Day Fish Assay (GD No. 150 Section C.3.2)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows elevated ♂ VTG, while existing *in vitro* data show a variety of responses consistent with ER interactions, and existing *in vivo* data show a variety of endpoints consistent with ER agonism.

Table 5.4.2.1: tert-OP data summary

21 Day Fish Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
♂ VTG↑ (NOEC = 27.8 µg/l) (Japanese Ministry of the Environment 2006). In a non-standard assay, hepatic ♂ VTG ↑ (NOEC = 27.8 µg tert-OP/l) (Nozaka *et al*. 2004).	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	TG 229 gave the following results: ♂ VTG↑, ♂ secondary sexual characteristics ↓, fecundity and fertility ↓. LOECs for effects on VTG in the range 0.6-37 µg/l, and for effects on secondary sexual characteristics in the range 0.6-113 µg/l. LOECs for reduced fecundity and fertility were 113 µg/l. (Biever *et al*. 2007). A non-standard assay in which only ♂♂ were exposed to tert-OP gave a NOEC for ↑♂ VTG of 20 µg/l and for ↓ fecundity of <20 µg/l (Gronen *et al*., 1999). These results support those obtained with TG 230.

21 Day Fish Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006).	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	Non-standard short-term studies with fish show ♂ and ♀ VTG ↑ (or ♂ VTG mRNA transcripts ↑; and/or ♂ ZPP/ZRP ↑) in a large range of species after either tert-OP injection, feeding or ambient exposure. Ambient water NOECs for these effects ranged from 1.6 to 10 µg/l. (Andreassen & Korsgaard 2000; Andreassen *et al*. 2005; Bangsgaard *et al*. 2006; Bjerregaard *et al*. 2008; Brian *et al*. 2005; Chikae *et al*. 2003; Genovese *et al*. 2011; Huang & Wang, 2001; Jespersen *et al*. 2010; Jobling *et al*. 1996; Karels *et al*. 2003; Knudsen *et al*. 1998; Madsen *et al*. 2002, 2003, 2006; Ortiz-Zarragoitia & Cajaraville 2005; Pait & Nelson 2003; Pedersen *et al*. 1999, 2003; Rasmussen & Korsgaard 2004; Routledge *et al*. 1998; Van den Belt *et al*. 2003; Zhang *et al*. 2010). Some of these studies also reported reductions in milt volume, GSI, smolting ability, growth and mating behaviour, or
	ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
		Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	
	AR antagonism: AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).		

21 Day Fish Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	abnormalities in testicular histology and spermatocrit. The results of these studies largely support the results obtained with TG 230.
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).		
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		A non-standard short-term assay with adult male clawed frogs failed to induce VTG after i.p. injection (100 mg/kg/week), although this response was triggered by E2 (Van Wyk *et al*. 2003). ♀ and ♂ bullfrog tadpoles exposed short-term in a non-standard test to tert-OP underwent early gonadal differentiation (NOEC < 0.206 µg/l) (Mayer *et al*. 2003).
	PR binding (weak affinity) (Laws *et al* 2000)		
			Short term exposures of zebra finch chicks to orally dosed tert-OP (206 mg/kg body wt.) caused increases in oviduct weight

21 Day Fish Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			(Millam *et al*. 2002).

Table 5.4.2.2: Conclusions about 21 Day Fish Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
Strong evidence for *in vivo* endocrine activity in fish and other organisms	Consider performing a fish lifecycle test, especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	A standard life cycle test with medaka (Japanese Ministry of the Environment 2006) gave NOECs for induction of ♂ VTG of 4.3 µg/l, for induction of testis-ova of 9.9 µg/l. However, there were no effects on growth, GSI or time to hatching, and equivocal effects on hatchability at 82 µg/l. In a life cycle test with zebrafish, Segner *et al*. (2003) reported reduced fertilisation success with a 78 d EC50 of 28 µg/l. In another test with zebrafish, Wenzel *et al*. (2001) did not show effects on sex ratio, but reported reduced growth, fecundity and fertility with NOECs of 12 µg/l. In non-standard long-term tests, Gray *et al* (1999b) showed a significant reduction in sexual behaviour and overall reproductive success of medaka (NOEC = 10 µg/l) and a reduction in fertilisation success (NOEC <10 µg/l); and Robinson *et al*. 2004 showed that sand goby nuptial colouration and development of sperm duct glands was inhibited after 6 months at 28 µg/l; On the other hand, Toft & Baatrup (2001; 2003) failed to demonstrate altered sex ratios or

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
		reduced reproductive capability in guppies at concentrations up to 900 µg/l. In summary, some fish life cycle tests showed testis-ova and adverse effects on overall reproductive success, the lowest NOEC for such adverse impacts being 9.9 µg/l. The various NOECs were in the same range, or higher, than the NOECs observed in standard and non-standard versions of TG 230, and confirm that this screening assay gave an alert for effects on reproduction.

Table 5.4.2.3: Other considerations and conclusions for octylphenol. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for tert-OP
An alternative approach would be to deploy the FSDT, especially if sexual development is expected to give a response at lower concentrations than reproduction.	In a standard FSDT (OECD, 2011), zebrafish gave NOECs of 9.5-17.6 µg/l for ♂VTG induction and 5.7-<13.8 µg/l for ♀-biased sex ratio and Japanese medaka gave NOECs of 6.2 -31.7 µg/l for ♂and ♀ VTG induction and <11.2-23.5 µg/l for ♀-biased sex ratio. Three-spined sticklebacks were a little less sensitive with NOECs of 22.5->41.9 µg/l for ♂VTG induction and >41.9-66.0 µg/l for undifferentiated biased sex ratio. A test with medaka (Knörr & Braunbeck 2002) gave a NOEC of 20 µg/l for ♀-biased sex ratio. A second test with medaka (Seki *et al*. 2003) gave a NOEC for ♀-biased sex ratio of 23.7 µg/l. In a non-standard test similar to the FSDT, Gray *et al*. (1999a) demonstrated a NOEC of <100 µg/l for testis-ova induction in medaka. However, Toft & Baatrup (2003) reported no effect on sex ratio in guppies at concentrations up to 200 µg/l, but the NOEC for adverse effects on sperm count and gonopodial length was 11.7 µg/l. In summary, ♀-biased sex ratio in 2 species (zebrafish and medaka) occurred with NOECs in the range 5.7-23.7 µg/l. These NOECs were in a similar range to NOECs for adverse effects derived from full lifecycle tests, and equal to or higher than NOECs derived from TG 229. On the other hand, a third species

Other considerations (as given in BB)	Conclusions for tert-OP
	(guppy) did not show biased sex ratio at concentrations up to 200 µg/l.
The decision about whether to conduct a FLCTT or MMGT may be driven primarily by the bioaccumulative properties of the chemical – a one generation test (*e.g.* F0 eggs to F1 fry) will generally be sufficient if the chemical is not expected to be transferred to the fry via the eggs.	No MMGT data are available. However, tert-OP is not strongly bioaccumulative, and may therefore not be significantly less potent in the FLCTT compared with the MMGT.

54. Overall conclusions about 21 Day Fish Assay and existing data: In the light of estrogenic effects on ♂ VTG demonstrated in TG 230, the BB conclusion that adverse effects on reproduction might occur, and the advice to conduct a fish full life cycle or fish sexual development test, were vindicated because these tests (in some species) revealed adverse effects on sex ratio and a variety of reproductive endpoints including overall reproductive success. The NOECs for adverse effects in both the partial and full fish lifecycle tests were similar to, or higher, than NOECs reported for TG 230. The limited data from amphibians did not strongly predict the estrogenic effects in fish, but limited data from birds and abundant data from mammals were both predictive of such effects.

5.4.3 Variant of OECD TG 230: Androgenised Female Stickleback Screen (AFSS) (GD No. 150 Section C.3.3)

No data available.

5.4.4 Fish sexual development test (FSDT) (TG 234) (GD No. 150 Section C.3.4)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows elevated ♂ VTG and ♀-biased sex ratio, while existing *in vitro* data show a variety of responses consistent with ER interactions, and existing *in vivo* data show a variety of endpoints consistent with ER agonism.

Table 5.4.4.1: tert-OP data summary

Fish Sexual Development Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
In a standard FSDT (OECD, 2011), zebrafish gave NOECs of 9.5-17.6 µg/l for	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000;	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle	TG 229 gave the following results: ♂ VTG↑, ♂ secondary sexual characteristics ↓, fecundity and fertility ↓. LOECs for

Fish Sexual Development Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
♂VTG induction and 5.7-<13.8 µg/l for ♀-biased sex ratio and Japanese medaka gave NOECs of 6.2 - 31.7 µg/l for ♂and ♀ VTG induction and <11.2-23.5 µg/l for ♀-biased sex ratio. Three-spined sticklebacks were a little less sensitive with NOECs of 22.5->41.9 µg/l for ♂VTG induction and >41.9-66.0 µg/l for undifferentiated biased sex ratio. A test with medaka (Knörr & Braunbeck 2002) gave a NOEC of 20 µg/l for ♀-biased sex ratio. A second test with medaka (Seki *et al*. 2003) gave a NOEC for ♀-biased sex ratio of 23.7 µg/l. In a non-standard test similar to the	Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	effects on VTG in the range 0.6-37 µg/l, and for effects on secondary sexual characteristics in the range 0.6-113 µg/l. LOECs for reduced fecundity and fertility were 113 µg/l. (Biever *et al*. 2007). A non-standard version of TG 229 in which only ♂♂ were exposed to tert-OP gave a NOEC for ↑♂ VTG of 20 µg/l and for ↓ fecundity of <20 µg/l (Gronen *et al*., 1999).
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009). TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)		TG 230: ♂ VTG↑ (NOEC = 27.8 µg/l) (Japanese Ministry of the Environment 2006). In a non-standard assay, hepatic ♂ VTG ↑ (NOEC = 27.8 µg tert-OP/l) (Nozaka *et al*. 2004).

Fish Sexual Development Test data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
FSDT, Gray *et al*. (1999a) demonstrated a NOEC of <100 µg/l for testis-ova induction in medaka. However, Toft & Baatrup (2003) reported no effect on sex ratio in guppies at concentrations up to 200 µg/l, but the NOEC for adverse effects on sperm count and gonopodial length was 11.7 µg/l.	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	Non-standard short-term studies with fish show ♂ and ♀ VTG ↑ (or ♂ VTG mRNA transcripts ↑; and/or ♂ ZPP/ZRP ↑) in a large range of species after either tert-OP injection, feeding or ambient exposure. Ambient water NOECs for these effects ranged from 1.6 to 10 µg/l. (Andreassen & Korsgaard 2000; Andreassen *et al*. 2005; Bangsgaard *et al*. 2006; Bjerregaard *et al*. 2008; Brian *et al*. 2005; Chikae *et al*. 2003; Genovese *et al*. 2011; Huang & Wang, 2001; Jespersen *et al*. 2010; Jobling *et al*. 1996; Karels *et al*. 2003; Knudsen *et al*. 1998; Madsen *et al*. 2002, 2003, 2006; Ortiz-Zarragoitia & Cajaraville 2005; Pait & Nelson 2003; Pedersen *et al*. 1999, 2003; Rasmussen & Korsgaard 2004; Routledge *et al*. 1998; Van den Belt *et al*. 2003; Zhang *et al*. 2010). Some of these studies also reported reductions in milt volume, GSI, smolting ability, growth and mating behaviour, or
	AR antagonism: AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).		
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).		

Fish Sexual Development Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			abnormalities in testicular histology and spermatocrit.
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	In partial lifecycle non-standard fish tests, results reported range from no effects on sexual development (Carlson *et al*. 2000), to reduced courtship behaviour, reduced fertilisation success, reduced overall reproductive success, testicular abnormalities, lower GSI, reduced body colouration, reduced testosterone and increased estradiol. NOECs in ambient water for these effects range from <10 to 100 µg/l (Gray *et al*. 1999b; Kinnberg & Toft 2003; Mills *et al*. 2001; Toft & Baatrup 2001; Vázquez *et al*. 2009; Zaroogian *et al*. 2001). The results of these non-standard partial lifecycle tests show that, in addition to abnormal sex ratios, a variety of effects on sexual development and reproductive success may occur.
	PR binding (weak affinity) (Laws *et al* 2000)		
		In a non-standard female PP assay VO was advanced by 3.2 days at oral doses of 200	A non-standard short-term assay with adult male clawed frogs failed to induce VTG

Fish Sexual Development Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		mg/kg/day. No other parameters were determined.	after i.p. injection (100 mg/kg/week), although this response was triggered by E2 (Van Wyk *et al*. 2003). ♀ and ♂ bullfrog tadpoles exposed short-term in a non-standard test to tert-OP underwent early gonadal differentiation (NOEC < 0.206 µg/l) (Mayer *et al*. 2003).
		Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c.	In a non-standard partial lifecycle test with clawed frog (*X. laevis*), effects observed included increased numbers of female phenotypes (NOEC = <2.1 µg/l) (Kloas *et al*. 1999). This result is similar to the ♀-biased sex ratios seen with the FSDT (TG 234), with an equivalent or lower NOEC. However, in a partial lifecycle test with the closely related *X. tropicalis* (Porter *et al*. 2011), there were no effects on sex ratio although some males developed oviducts (NOEC = 11 µg/l).

Fish Sexual Development Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
			Short term exposures of zebra finch chicks to orally dosed tert-OP (206 mg/kg body wt.) caused increases in oviduct weight (Millam *et al*. 2002).
			Partial lifecycle tests with birds produced results showing no impact on reproductive success or song control nuclei at 20-206 mg/kg body wt.(Millam *et al*. 2001; Quaglino *et al*. 2002). These results are not predictive of effects seen in the FSDT, but it should be noted that exposures were not continuous.

Table 5.4.4.2: Conclusions about Fish Sexual Development Test and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
Strong evidence for adverse effects in fish and other organisms by an endocrine mechanism.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish	A standard life cycle test with medaka (Japanese Ministry of the Environment 2006) gave NOECs for induction of ♂ VTG of 4.3 µg/l, for induction of testis-ova of 9.9 µg/l. However, there were no effects on growth, GSI or time to

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
	lifecycle test should be considered.	hatching, and equivocal effects on hatchability at 82 µg/l. In a life cycle test with zebrafish, Segner *et al*. (2003) reported reduced fertilisation success with a 78 d EC50 of 28 µg/l. In another test with zebrafish, Wenzel *et al*. (2001) did not show effects on sex ratio, but reported reduced growth, fecundity and fertility with NOECs of 12 µg/l. In non-standard long-term tests, Gray *et al* (1999b) showed a significant reduction in sexual behaviour and overall reproductive success of medaka (NOEC = 10 µg/l) and a reduction in fertilisation success (NOEC <10 µg/l); and Robinson *et al*. 2004 showed that sand goby nuptial colouration and development of sperm duct glands was inhibited after 6 months at 28 µg/l; On the other hand, Toft & Baatrup (2001; 2003) failed to demonstrate altered sex ratios or reduced reproductive capability in guppies at concentrations up to 900 µg/l. In summary, some fish life cycle tests showed testis-ova and adverse effects on overall reproductive success, the lowest NOEC for such adverse impacts being 9.9 µg/l. The various lifecycle NOECs were in the same range as the NOECs observed in standard and non-standard versions of TG 234, and confirm that this partial lifecycle test was predictive of long-term effects on reproduction.

Table 5.4.4.3: Other considerations and conclusions for octylphenol. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for octylphenol.

Other considerations (as given in BB)	Conclusions for tert-OP
If the FSDT was only performed with 3 test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case the FSDT would be less responsive than a lifecycle test.	In this case, tert-OP appears to be of high toxicity to sexual development, and it is of similar sensitivity in both the FSDT and FFLCT.
A decision about whether to conduct FLCTT or MMLC may be driven primarily by the bioaccumulative properties of the chemical – a one generation test (*e.g.* F0 eggs to F1 fry) will generally be sufficient if the chemical is not expected to be transferred to the fry via the eggs.	No MMGT data are available. However, OP is not strongly bioaccumulative, and may therefore not be significantly less potent in the FLCTT compared with the MMGT.

55. Overall conclusions about Fish Sexual Development Test and existing data: In the light of estrogenic effects on ♂ VTG and in particular on sex ratio demonstrated in TG 234, the BB conclusion that there is strong evidence for adverse effects in fish by an endocrine mechanism, and the advice to conduct a fish life cycle test (if further data are considered necessary), were vindicated because several full lifecycle tests demonstrated a range of impacts on various aspects of reproductive success and associated endpoints such as testis-ova and abnormal sexual behaviour. The lowest NOECs for adverse effects on reproduction in fish full lifecycle tests (~10 µg/l) were similar to, or slightly lower, than the lowest NOECs reported for TG 234 (5.7-23.7 µg/l for effects on sex ratio) . The partial life cycle data from amphibians varied between species. While a study with one species of clawed frog (*X. laevis*) showing a feminisation of the sex ratio with a NOEC of <2.1 µg/l, another with *X. tropicalis* showed no effects on sex ratio but growth of oviducts in males (NOEC = 11 µg/l), thus partially supporting predictions based on FSDT data. The long-term exposure data from rodents was also supportive of predictions based on TG 234. However, partial lifecycle test data from birds failed to show effects on reproduction at i.p. doses of 20-206 mg/kg body wt.

5.4.5 Fish Lifecycle Toxicity Test (FLCTT) (USEPA OPPTS 850.1500) (GD No. 150 Section C.3.5)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows testis-ova induction, depressed fecundity and fertility, and increased time to spawning, while existing *in vitro* data show a variety of responses consistent with ER interactions, and existing *in vivo* data show a variety of endpoints consistent with ER agonism.

Table 5.4.5.1: tert-OP data summary

Fish Lifecycle Toxicity Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
In a lifecycle test with medaka (Japanese Ministry of the Environment 2006), the NOEC for induction of testis-ova was 9.9 µg/l, but there were no other significant effects on reproductive endpoints. In a test with zebrafish (Segner *et al*. 2003), fertilisation success was reduced (78 d EC50 = 28 µg/l). In another test with zebrafish (Wenzel *et al*. 2001), there were reductions in growth, fecundity and fertility, and an increase in time to first spawning (NOEC = 12 µg/l).	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	TG 229 gave the following results: ♂ VTG↑, ♂ secondary sexual characteristics ↓, fecundity and fertility ↓. LOECs for effects on VTG in the range 0.6-37 µg/l, and for effects on secondary sexual characteristics in the range 0.6-113 µg/l. LOECs for reduced fecundity and fertility were 113 µg/l. (Biever *et al*. 2007).
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	A non-standard version of TG 229 in which only ♂♂ were exposed to OP gave a NOEC for ↑♂ VTG of 20 µg/l and for ↓ fecundity of <20 µg/l (Gronen *et al*., 1999).
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et*	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day	TG 230: ♂ VTG↑ (NOEC = 27.8 µg/l) (Japanese Ministry of the Environment 2006). In a non-standard assay, hepatic ♂ VTG ↑ (NOEC = 27.8 µg tert-OP/l) (Nozaka *et al*. 2004).

Fish Lifecycle Toxicity Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	al. 1994)	showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	Non-standard short-term studies with fish show ♂ and ♀ VTG ↑ (or ♂ VTG mRNA transcripts ↑; and/or ♂ ZPP/ZRP ↑) in a large range of species after either tert-OP injection, feeding or ambient exposure. Ambient water NOECs for these effects ranged from 1.6 to 10 µg/l. (Andreassen & Korsgaard 2000; Andreassen *et al*. 2005; Bangsgaard *et al*. 2006; Bjerregaard *et al*. 2008; Brian *et al*. 2005; Chikae *et al*. 2003; Genovese *et al*. 2011; Huang & Wang, 2001; Jespersen *et al*. 2010; Jobling *et al*. 1996; Karels *et al*. 2003; Knudsen *et al*. 1998; Madsen *et al*. 2002, 2003, 2006; Ortiz-Zarragoitia & Cajaraville 2005; Pait & Nelson 2003; Pedersen *et al*. 1999, 2003; Rasmussen & Korsgaard 2004; Routledge *et al*. 1998; Van den Belt *et al*. 2003; Zhang *et al*. 2010). Some of these studies also reported reductions in milt

Fish Lifecycle Toxicity Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			volume, GSI, smolting ability, growth and mating behaviour, or abnormalities in testicular histology and spermatocrit.
	AR antagonism: AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	In partial lifecycle non-standard fish tests, results reported range from no effects on sexual development (Carlson *et al*. 2000), to reduced courtship behaviour, reduced fertilisation success, reduced overall reproductive success, testicular abnormalities, lower GSI, reduced body colouration, reduced testosterone and increased estradiol. NOECs in ambient water for these effects range from <10 to 100 µg/l (Kinnberg & Toft 2003; Mills *et al*. 2001; Toft & Baatrup 2001; Vázquez *et al*. 2009; Zaroogian *et al*. 2001). The results of these non-standard partial lifecycle tests show that, in addition to abnormal sex ratios, a variety of effects on sexual development and reproductive success may occur.
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on	Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of	In a non-standard fish lifecycle test with medaka (Gray *et al*. 1999b), overall

Fish Lifecycle Toxicity Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	reproductive success was reduced (NOEC = 10 µg/l), and offspring had developmental problems (NOEC <10 µg/l).
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	A non-standard short-term assay with adult male clawed frogs failed to induce VTG after i.p. injection (100 mg/kg/week), although this response was triggered by E2 (Van Wyk *et al*. 2003). ♀ and ♂ bullfrog tadpoles exposed short-term in a non-standard test to tert-OP underwent early gonadal differentiation (NOEC < 0.206 µg/l) (Mayer *et al*. 2003).
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		In a non-standard partial lifecycle test with clawed frog (*X. laevis*), effects observed included increased numbers of female phenotypes (NOEC = <2.1 µg/l) (Kloas *et al*. 1999). This result is similar to the ♀-biased sex ratios seen with the FSDT (TG 234), with an equivalent or lower NOEC. However, in a partial lifecycle test with the closely related *X. tropicalis* (Porter *et al*. 2011), there were

Fish Lifecycle Toxicity Test data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			no effects on sex ratio although some males developed oviducts (NOEC = 11 µg/l).
	PR binding (weak affinity) (Laws *et al* 2000)	In a non-standard female PP assay VO was advanced by 3.2 days at oral doses of 200 mg/kg/day. No other parameters were determined.	Short term exposures of zebra finch chicks to orally dosed tert-OP (206 mg/kg body wt.) caused increases in oviduct weight (Millam *et al*. 2002).
			Partial lifecycle tests with birds produced results showing no impact on reproductive success or song control nuclei at 20-206 mg/kg body wt.(Millam *et al*. 2001; Quaglino *et al*. 2002). These results are not predictive of effects seen in the FFLCT, but it should be noted that exposures were not continuous.

Table 5.4.5.2: Conclusions about Fish Life Cycle Toxicity Test and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
The test chemical is almost certainly an ED if the modality identified in existing screens/tests can be plausibly linked to the affected endpoint. In this case, the modality already identified (weak estrogenicity) is indeed clearly linked to most of the	Further evidence is probably not required. In this case, there is good evidence that reproductive success is impacted in more than one fish species, and effects observed in amphibians could potentially affect reproductive success,	n/a

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
responding endpoints.	although lifecycle tests with amphibians have not been conducted.	

Table 5.4.5.3: Other considerations and conclusions for octylphenol. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for octylphenol.

Other considerations (as given in BB)	Conclusions for tert-OP
If the affected endpoint in the FLCTT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED.	In this case, it can be plausibly argued that the effects observed in various lifecycle and partial lifecycle tests (ranging from mechanistic endpoints such as VTG induction and ovo-testis, to apical endpoints such as female-biased sex ratios and reduced reproductive success) are all caused by exposure to tert-OP acting as a weak estrogen. It is possible that some of these effects could be caused by non-estrogenic mechanisms,but the overall weight of evidence points to an estrogenic MOA.
The FLCTT is unlikely to detect epigenetic effects. If these are suspected, an MMGT may reveal them.	No data are available for the MMGT.

56. Overall conclusions about Fish Life Cycle Toxicity Test and existing data: Although some reproductive endpoints did not respond to tert-OP in some fish species, there were clear and substantial impacts on various aspects of reproductive success and associated mechanistic endocrine biomarkers in several species. The weight of evidence clearly pointed to a causal link between the estrogenic mode of action of tert-OP and these reproductive effects. The lowest NOECs for effects on fish reproduction were in the range <10-12 µg/l, *i.e.* no lower than the lowest NOECs reported for the FSDT (TG 234) or the FSTRA (TG 229). These data would be sufficient for the conduct of a reliable environmental risk assessment with respect to fish populations, and further data on fish would not be necessary. They are supported by the wealth of evidence from mammalian test systems, and by the limited data from amphibians. Limited data for birds do not, however, suggest that tert-OP damages reproductive success in this group, although it does appear to cause some estrogenic effects. Avian lifecycle data are not available.

5.4.6 OECD TG 231: Amphibian Metamorphosis Assay (AMA) (GD No. 150 Section C.3.6)

No data available.

5.4.7 OECD TG 206: Avian Reproduction Test (GD No. 150 Section C.3.7)

No data available.

5.5 4-tert-Octylphenol Case Study Results: Validated Mammalian *in vivo* Assays

5.5.1 OECD TG 440: Uterotrophic Bioassay in Rodents (UT assay) (Including GD on the Use of the Assay to Screen for Anti-Estrogenicity) (GD No. 150 Section C.4.1)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that tert-OP was positive for ER agonism in UT assays, while existing *in vitro* data show a variety of responses including ER binding and agonism, AR binding and antagonism, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.5.1.1: tert-OP data summary

Uterotrophic Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism. Increases in uterine weights occurred at oral doses of 100 mg/kg/day and above (no effects at 50 mg/kg/day). Administration via the s.c. route resulted in uterine weight increases at doses of 50 mg/kg/day and above. (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a)	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish.	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	

Uterotrophic Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	(Fu *et al*. 2007; Gale *et al*. 2004)		
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006;	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).		
	Effects in thyroid assays.		

Uterotrophic Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009). Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).	Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	PR binding (weak affinity) (Laws *et al* 2000)	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	

Table 5.5.1.2: Conclusions about Uterotrophic Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for Tert-OP
Strong evidence that tert-OP has E activity with (potential for) adverse effects via ER mechanism.	Perform assay from upper levels *e.g.* female pubertal assay (level 4)	Female PP assay : positive ER agonism. A non-standard assay with a single endpoint (VO) resulted in age of VO being advanced by 3.2 days at oral doses of 200 mg/kg/day. (Laws *et al*, 2000).
	or ext-1 or 2-gen assay (level 5).	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.

Table 5.5.1.3: Other considerations and conclusis for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	**conclusions for tert-OP**
If existing data are from level 4 or 5 (or less sensitive assays) then there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The "mammalian effects of concern" are provided by some effects in TG 407 studies (level 4) (Bian *et al* 2006; Hossaini *et al* 2003; Laws *et al* 2000) whilst other TG 407 and TG 408 studies showed no effects (CIPT, 1994; HRC, 1994; Sahambi *et al*, 2010). The lack of effects in the 2-gen assay (TG 416) (Tyl *et al* 1999) indicates that although the tert-OP is an estrogen agonist *in vivo* in the UT assay, it does not have adverse effects on reproduction or development in intact animals. The data where s.c. administration was used did not give consistent results. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention).
Consider route of exposures for UT assay and existing effects data and possible implications of ADME characteristics of the chemical.	Tert-OP appears to produce endocrine effects at a lower dose, when administered s.c rather than orally. This is consistent with rapid first pass metabolism via the oral route and the potential to achieve higher internal doses when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	Tert-OP is metabolised by hydroxylation, glucuronidation and sulphation. The glucuronide metabolite had no activity when tested in ER and AR transactivation assays for agonism and antagonism. This indicates that glucuronide conjugation is a detoxification reaction (Moffat et al, 2001).
A positive result could have arisen from other (EATS or non-EATS) mechanisms.	Tert-OP is an AR antagonist (*in vitro*), there is. some evidence of steroidogenesis disruption (Murono *et al* 2000, 2001, 2002), weak binding to PR (Laws *et al* 2000) and possible thyroid disruption (*in vitro*) (Ghisari *et al* 2009). The positive result in the UT assay is however, unlikely to have arisen via these other mechanisms.

57. Overall conclusions about Uterotrophic Assay and existing data: next steps and other considerations provide a logical course of action to follow. The agonist response in the UT assay demonstrates that the ER agonism response *in vitro* also has the potential to occur *in vivo*. The oral dose at which the UT response

was seen corresponded with the oral dose at which earlier age at VO occurred in the (non-standard) peripubertal assay. Interaction with other endocrine systems was also shown *in vitro*. The combined dataset provides evidence that tert-OP can interact with ER and other endocrine systems. In mammalian species, the available evidence from a level 5 assay indicates that this interaction does not result in effects on reproduction when administration is via the oral route. Other routes, where a higher internal dose may be achieved, may give different results. The effects seen in the UT assay may give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different. In fact in fish and amphibians, a variety of effects on sexual development and reproduction are caused by tert-OP.

5.5.2 OECD TG 441: Hershberger Bioassay in Rats (H Assay) (Including OECD GD for Weanling Hershberger Bioassay) (GD No. 150 Section C.4.2)

Scenario J : Result -, existing *in vitro* data +, existing *in vivo* data +

Scenario J is appropriate because tert-OP was negative in Hershberger (H) assays for AR agonism and antagonism, with administration via the oral route. Existing *in vitro* data show a variety of responses including ER binding and agonism, AR binding and antagonism, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights in some TG 407 assays are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.5.2.1: tert-OP data summary

Hershberger Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day. (Yamasaki *et al* 2003)	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006).	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	

Hershberger Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)		
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).		

Hershberger Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).	Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)		

Table 5.5.2.2: Conclusions about Hershberger Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
No evidence that tert-OP has A/ anti-A activity via AR or 5-alpha reductase inhibition *in vivo* when tested in the H assay. Route of exposure, metabolic differences or doseage differences may explain the differences between H assay and	Perform AR transcriptional assay or binding assay with added metabolising system.	Tert-OP binds to AR and is an antagonist in the AR STTA assay, therefore conduct of these assays with additional metabolizing systems is unnecessary. It is likely that the weak effects observed in these assays and/or metabolism of tert-OP *in vivo* are the reasons for the negative result

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
existing *in vitro*/*in vivo* studies. Reductions in SATs were observed at doses 2-fold higher than those used in the H assay (400 mg/kg/day and above) in TG 407 assays (Bian *et al*, 2006; Hossaini *et al* 2003). Effects seen in existing studies are via non-AR/5-alpha reductase mechanism. It is also possible that the effects on SATs observed in the existing studies are due to ER agonism.		in the H assay. ADME studies have indicated that tert-OP undergoes rapid first pass metabolism.

Table 5.5.2.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	**conclusions for tert-OP**
If existing data are from level 4 or 5 (or less sensitive assays) then there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The "mammalian effects of concern" are provided by some effects in TG 407 studies (level 4) (Bian *et al* 2006; Hossaini *et al* 2003; Laws *et al* 2000) whilst other TG 407 and TG 408 studies showed no effects (CIPT, 1994; HRC, 1994; Sahambi *et al*, 2010). UT assays were also positive, consistent with ER agonist activity (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a). A robust 2-gen (TG 416) study however, showed that tert-OP had no effects on endocrine endpoints, reproduction or development in intact animals (Tyl *et al* 1999). The data where s.c. administration was used did not give consistent results. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention).
Consider route of exposures for H assay and existing effects data and possible implications of ADME characteristics of the chemical.	Higher doses were used in TG 407 assays where reductions in SATs were observed (Bian *et al*, 2006; Hossaini *et al* 2003) compared to in the H assay. This may explain the different results. tert-OP also undergoes rapid first pass metabolism when given orally and this may also account for the negative H assay in the presence of positive AR *in vitro* assays.
Effects seen in existing studies may be in a more sensitive life stage	This is unlikely to be the case as the 2-gen (TG 416) study was negative.
Check data on chemical analogues. Further mechanistic studies would help determine MoA.	Other alkyl phenols (p-cumyl phenol and nonylphenol) were also negative in the H assay (Yamasaki *et al*, 2003).

58. Overall conclusions about Hershberger Assay and existing data: next steps and other considerations provide a logical course of action to follow. There are a number of possible explanations for the negative H assay in the presence of positive *in vitro* AR-based assays. These were prompted by the guidance and

further data (*e.g.* results on analogues) supports these considerations. Interaction with other endocrine systems was also shown *in vitro*. The combined dataset indicates that the interaction with AR *in vitro* does not result in effects manifested *in vivo*. However, tert-OP can interact with ER in vivo, as demonstrated by the positive agonist response in the UT assay. In mammalian species, the available evidence from a level 5 assay indicates that this interaction does not result in effects on reproduction when administration is via the oral route. In contrast, in fish and amphibians, a variety of effects on sexual development and reproduction are probably caused by the estrogenic effects of tert-OP..

5.5.3. Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats (Male PP Assay) (US EPA OPPTS 890.1500) (GD No. 150 Section C.4.3)

No data available

5.5.4. Pubertal Development and Thyroid Function Assay in Peripubertal Female Rats (Female PP Assay) (US EPA OPPTS 890.1450) (GD No. 150 Section C.4.4)

The only assay available was non-standard with only one endpoint and therefore it was not considered appropriate to be used as a case study.

5.5.5. OECD TG 407: Repeated Dose 28 Day Oral Toxicity Study in Rodents (GD No. 150 Section C.4.5)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because tert-OP gave positive results in non-standard TG 407 assays, although it is acknowledged that some data were contradictory (see Column 1 in Table 5.5.5.1). Existing *in vitro* data show a variety of responses including ER binding and agonism, AR binding and antagonism, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data have some positive and some negative results. The positive results in the UT assays and advancement of VO in a PP assay are consistent with effects on ER and therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.5.5.1: tert-OP data summary

Repeated Dose 28 Day Rodent Oral Toxicity Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
In standard assays where tert-OP was administered up to 300 mg/kg/day, there were no effects on endocrine endpoints in male or female rats. In non-standard assays, reductions in some male SAT weights were seen at doses of 400 mg/kg/day and above. Some histopathological changes in testes and reduced sperm counts were seen at 450 mg/kg/day. In one study, disturbances of the estrus cycle were seen at 200 mg/kg/day whilst in another study no effects in female rats were observed at doses up to 125 mg/kg/day. Although the data are somewhat conflicting, the overall conclusion is that at high doses there are effects on both apical endpoints and indicators of hormonal activity	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	No data
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
	AR binding. (Paris *et al* 2002; Li *et al*	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage	

Repeated Dose 28 Day Rodent Oral Toxicity Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
(Outcome 1 in guidance document). (CIPT 1994; HRC 1994; Laws *et al* 2000; Bian *et al* 2006; Sahambi *et al* 2010)	2010).	had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Tert-OP was positive in both the immature and the ovariectomized rat assay	

Repeated Dose 28 Day Rodent Oral Toxicity Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)	In a non-standard female PP assay VO was advanced by 3.2 days at oral doses of 200 mg/kg/day. No other parameters were determined.	

Table 5.5.5.2: Conclusions about Repeated Dose 28 day Rodent Oral Toxicity Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay Result for tert-OP
Increased evidence that tert-OP has E and possibly anti-A and/or S activity (at high dose levels).	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.

Table 5.5.5.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for tert-OP
If existing data are from level 5 then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	The "mammalian effects of concern" in this case are provided by the positive UT assays. Other assays: H assays and TG 408 studies are negative. The lack of effects in the 2-gen assay (TG 416) (Tyl *et al* 1999) indicates that although the tert-OP is an estrogen agonist *in vivo* in the UT assay, it does not have adverse effects on endocrine endpoints, reproduction or development in intact animals when administered at doses up to 111-369 (dependent on the stage of the study). The positive results in TG 407 assays were generally obtained at doses above this. The data where s.c. administration was used did not give consistent results. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention).
Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints.	Perturbation of the estrus cycle was observed at 200 mg/kg/day in one study (Laws *et al* 2000) whilst in other studies there were no effects on apical endpoints at doses up to 300 mg/kg/day (Sahambi *et al* 2010).
Effects on apical endpoints alone may indicate EATS modalities or other mechanisms.	Consideration is valid. The effects observed could have arisen via E or anti-A modalities.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Tert-OP appears to produce endocrine effects at a lower dose, when administered s.c rather than orally. This is consistent with rapid first pass metabolism via the oral route and the potential to achieve higher internal doses when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure. Received doses may explain the differences between the positive and negative TG 407 studies and also between the positive TG 407 studies and the negative TG 416 study.

59. Overall conclusions about Repeated Dose 28 day Rodent Oral Toxicity Assay and existing data: next steps and other considerations provide a logical course of action to follow. The effects on endocrine

endpoints occurring at doses above 200 mg/kg/day may result from estrogen agonism (as seen in the the positive level 3 UT assay). Interaction with other endocrine systems was also shown *in vitro* and therefore other mechanisms of action may also have a role. The TG407 is an apical assay and therefore it is difficult to discern mechanism. In mammalian species, the available evidence from a level 5 assay indicates that any endocrine activity does not result in adverse effects on reproduction when administration is via the oral route. Other routes, where a higher internal dose may be achieved, may give different results.In fish and amphibians, a variety of effects on sexual development and reproduction are caused by tert-OP.

5.5.6 OECD TG 416: Two-Generation Reproduction Toxicity Study (GD No. 150 Section C.4.6)

Scenario J : Result -, existing *in vitro* data +, existing *in vivo* data +

Scenario J is appropriate because tert-OP was negative in a robust 2-generation study with administration via the oral route. It is acknowledged that a smaller one generation study with administration via the s.c. route gave some positive responses (see Column 1 Table 5.5.6.1) but for the purposes of this case study the negative assay result scenario has been considered. Existing *in vitro* data show a variety of responses including ER binding and agonism, AR binding and antagonism, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights in some TG 407 assays are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.5.6.1: tert-OP data summary

Two-Generation Reproduction Toxicity Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
No effects on endocrine endpoints, reproduction or development were noted in a 2-generation study at dietary doses from 0.2-2000 ppm (up to 111-369 mg/kg/day depending upon the phase of the	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data

Two-Generation Reproduction Toxicity Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
study). Study was considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study (one generation) using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009). Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is		

Two-Generation Reproduction Toxicity Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).		
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)	In a non-standard female PP assay VO was advanced by 3.2 days at oral doses of 200 mg/kg/day. No other parameters were determined.	

Table 5.5.6.2: Conclusions about 2 Generation Reproduction Toxicity Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay Result for tert-OP
Tert-OP treatment produced no evidence of adverse effects on reproduction/development/endocrine organs in TG 416 at the doses tested. Effects seen in existing (lower level) studies do not lead to adverse outcome in level 5 assay. It is likely that metabolism and doseage	If test is to current TG 416 standards, no further testing needed. If not then consider supplemental testing, depending upon existing data. To further discern	The existing 2-gen study is considered to be of good quality although does not contain as many endocrine-sensitive endpoints as the new ext-1-gen assay (TG 443). Differences in dose levels and adminstration are

Conclusions for tert-OP	Next Step (as BB)	Assay Result for tert-OP
differences explain the difference from existing *in vitro*/ and *in vivo* data.	mechanism could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	likely to explain the differences between the results of Tyl *et al* (1999) and Yoshida *et al* (2001). Tert-OP undergoes rapid first pass metabolism via the oral route and therefore higher internal doses can be achieved when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.

Table 5.5.6.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for tert-OP
If existing data are from adequate *in vivo* studies such as 28d, 90d, chronic/carcinogenicity studies, than question why differences.	The "mammalian effects of concern" are provided by some effects in TG 407 studies (level 4) (Bian *et al* 2006; Hossaini *et al* 2003; Laws *et al* 2000) whilst other TG 407 and TG 408 studies showed no effects (CIPT, 1994; HRC, 1994; Sahambi *et al*, 2010). UT assays were also positive, consistent with ER agonist activity (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a). The data where s.c. administration was used did not give consistent results
Note that the ext-1 gen assay provides the most information on endocrine disruption.	An ext-1-gen assay (TG 443) would provide the most extensive evaluation of the endocrine disruption potential of tert-OP. However, the existing study is adequate.
Consider route of exposures and possible implications for ADME characteristics of the chemical with existing studies.	Higher doses were used in TG 407 assays where reductions in SATs were observed (Bian *et al*, 2006; Hossaini *et al* 2003) compared to in the 2-gen (TG 416) assay. This may explain the different results. tert-OP also undergoes rapid first pass metabolism when given orally and this may also account for the differences between the results of Tyle *et al* (1999) where dietary adminstration was used and Yoshida *et al* (2001) where s.c. adminstration was used.
Further mechanistic studies with metabolism may help determine MoA.	Not necessary as adequate mechanistic data exist.

Other considerations (as given in BB)	Conclusions for tert-OP

60. Overall conclusions about 2 Generation Reproduction Toxicity Assay and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence that tert-OP can interact with endocrine systems although the available evidence from the 2- Generation Reproduction Toxicity Assay indicates that any endocrine activity does not result in effects on reproduction when administration is via the oral route. ER agonism was, however, demonstrated *in vivo* in the UT assay. It is possible, therefore that administration of tert-OP by other routes, where a higher internal dose may be achieved, may give different results. It is also noted that tert-OP has not been investigated in the new extended 1-generation study (TG 443), the currently most comprehensive OECD TG on mammalian species for detection of sex hormone related endocrine disruption leading to adverse effects. Interestingly, in fish and amphibians, a variety of adverse effects on sexual development and reproduction are caused by tert-OP.

5.5.7 Extended One-Generation Reproductive Toxicity Study (draft OECD TG 443) (GD No. 150 Section C.4.7)

No data available

5.6 4-Tert-Octylphenol Case Study Results: *In vitro* Assays that Have Not Yet Completed Validation

5.6.1 The Human AR Transcriptional Activation Assay for Detection of Androgen (Ant)agonist-Activity of Chemicals (AR STTA) (GD No. 150 Section Annex 2.1)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows that tert-OP shows an antagonist response in AR transactivation assays, while existing *in vitro* data show a variety of responses including ER binding and ER agonism, AR binding, steroidogenesis disruption and possibly TH disruption. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.6.1.1: tert-OP data summary

Human AR Transcriptional Activation Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
Tert-OP antagonized activation of the reporter gene in the presence of R1881.	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005;	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003),	No data

Human AR Transcriptional Activation Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
IC50 was approximately 2µM. (Krüger *et al* 2008; Paris *et al* 2002).	Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	

Human AR Transcriptional Activation Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).		
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)		

Table 5.6.1.2: Conclusions about Human AR Transcriptional Activation Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for Tert-OP
Tert-OP antagonizes AR combined with effects on ER. Some possible effect on S and T. Potential for adverse effects via	Perform assay from upper levels *e.g.* H assay (level 3)	H assay : negative No agonistic or antagonistic effects were seen in the castrated rat assay at oral doses of 50 to 200

Conclusions for tert-OP	**Next Step (as BB)**	**Assay result for Tert-OP**
multiple mechanisms		mg/kg/day (Yamasaki *et al*, 2003).
	or fish screen (AFSS) (level 3)	No AFSS data are available.
	or male PP assay (level 4)	Not available.
	or ext-1 or 2-gen assays (level 5)	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.
	or partial/full fish life cycle tests (level 4/5).	A standard full life cycle test with medaka (Japanese Ministry of the Environment 2006) gave NOECs for induction of ♂ VTG of 4.3 µg/l, for induction of testis-ova of 9.9 µg/l. However, there were no effects on growth, GSI or time to hatching, and equivocal effects on hatchability at 82 µg/l. In a life cycle test with zebrafish, Segner *et al*. (2003) reported reduced fertilisation success with a 78 d EC50 of 28 µg/l. In another test with zebrafish, Wenzel *et al*. (2001) did not show effects on sex ratio, but reported reduced growth, fecundity and fertility with NOECs of 12 µg/l. In non-standard long-term tests, Gray *et al* (1999b) showed a significant reduction in sexual behaviour and overall reproductive success of medaka (NOEC = 10 µg/l) and a reduction in fertilisation success (NOEC <10 µg/l); and Robinson *et al*. 2004 showed that sand goby nuptial colouration and development of sperm duct glands was inhibited after 6 months at 28 µg/l; On the other hand, Toft & Baatrup (2001; 2003) failed to demonstrate

Conclusions for tert-OP	Next Step (as BB)	Assay result for Tert-OP
		altered sex ratios or reduced reproductive capability in guppies at concentrations up to 900 µg/l. In summary, some fish life cycle tests showed testis-ova and adverse effects on overall reproductive success, the lowest NOEC for such adverse impacts being 9.9 µg/l.

Table 5.6.1.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for Tert-OP
If existing data are from level 5 there may be sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Guideline level 5 study on tert-OP provides an adequate assessment by a relevant route (oral exposure) (Tyl *et al*, 1999). However this study lacks some endocrine endpoints present in more recent study designs (TG 443). The lack of effect via the oral route is supported by other oral studies in intact mammals. Studies using s.c. administration indicate that effects could be observed via this route (that may deliver a larger internal dose) but data are contradictory.
If existing data are from level 4 mammalian or wildlife assay then level 5 assay should provide more predictive information for endocrine disruption assessment.	No male PP assay is available and the female PP assay (level 4) on tert-OP does not provide sufficient data for ED assessment. Few endpoints were determined (Laws *et al*, 2000). Adequate level 5 type studies are therefore required. Even if full PP assays had been conducted it is likely that a level 5 study would be required so that a more comprehensive assessment could be made. Both Level 4 and 5 fish assays(Japanese Ministry of the Environment 2006; Segner *et al*., 2003; Wenzel *et al*. 2001; Gray *et al*., 1999a&b; Robinson *et al*., 2004; Toft & Baatrup, 2001& 2003; Knörr & Braunbeck 2002; Seki *et al*. 2003, and one non-standard Level 4 amphibian assay (Kloas *et al*., 1999) show adverse effects on sexual development and/or reproduction probably attributable to ER binding and not AR binding. However, a non-standard bird reproduction assay detected no effects (Millam *et al*. 2001)
If existing data are from H assay or AFSS then level 4 mammalian assay or fish screen (TG 229/230) will	H assay on tert-OP is negative for agonism and antagonism (Yamasaki *et al*, 2003). Level 4 PP

Other considerations (as given in BB)	Conclusions for Tert-OP
provide data on multiple modalities.	assay would provide more conclusive data especially given the conflicting results in repeat dosing studies. No data are available from an AFSS.
Consider route of exposures for existing effects data and possible implications of ADME characteristics of the chemical.	Tert-OP appears to produce endocrine effects at a lower dose, when administered s.c rather than orally. This is consistent with rapid first pass metabolism via the oral route and the potential to achieve higher internal doses when adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*.	Tert-OP is metabolised by hydroxylation, glucuronidation and sulphation. The glucuronide metabolite had no activity when tested in ER and AR transactivation assays for agonism and antagonism. This indicates that glucuronide conjugation is a detoxification reaction (Moffat et al, 2001).

61. Overall conclusions about Human AR Transcriptional Activation Assay and existing data: Next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence that the binding of tert-OP to AR results in inhibition of androgen-mediated reporter gene activation (antagonist response) *in vitro*. In the H assay (mammalian *in vivo* screening assay at level 3) tert-OP was negative indicating that the AR antagonism observed *in vitro* did not produce a similar response in vivo, under the conditions of the assay. . In mammalian species, the available evidence from a level 5 assay indicates that this interaction does not result in effects on reproduction when administration is via the the oral route Other routes, where a higher internal dose may be achieved, may give different results. Interestingly, the reverse is the case in fish and amphibians, where a variety of effects on sexual development and reproduction were caused by tert-OP.

5.7 4-Tert-Octylphenol Case Study Results: Wildlife *In vivo* Assays That Have Not Yet Completed Validation

5.7.1 Fish (Medaka) Multi-Generation Test (MMGT) (GD No. 150 Section Annex 2.2)

No data available

5.7.2 Larval amphibian growth and development assay (LAGDA) (GD No. 150 Section Annex 2.3)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows increased larval VTG and induction of oviducts in ♂♂, while existing *in vitro* data show a variety of responses consistent with ER interactions, and existing *in vivo* data show a variety of endpoints consistent with ER agonism.

Table 5.7.2.1: tert-OP data summary

Larval Amphibian Growth and Development Assay data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
No true LAGDA data with *X. laevis* are available, but a similar study has been conducted with *X. tropicalis* (Porter *et al*. 2011) exposed for 31 weeks from NF stage 46. Serum VTG increased in larvae (NOEC = 11 µg/l) but not in adults, and oviducts were observed in some adult ♂♂ (NOEC = 11 µg/l for ♂ oviduct weight), but there were no effects on sex ratio, sperm or oocyte counts or E2 or T titres. These results are interpreted to mean that some indicators of hormonal activity were positive, but not apical endpoints.	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	A non-standard short-term assay with adult male clawed frogs failed to induce VTG after i.p. injection (100 mg/kg/week), although this response was triggered by E2 (Van Wyk *et al*. 2003). ♀ and ♂ bullfrog tadpoles exposed short-term in a non-standard test to tert-OP underwent early gonadal differentiation (NOEC < 0.206 µg/l) (Mayer *et al*. 2003).
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	In a non-standard partial lifecycle test with clawed frog (*X. laevis*), effects observed included increased numbers of female phenotypes (NOEC = <2.1 µg/l) (Kloas *et al*. 1999).

Larval Amphibian Growth and Development Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	The TG 229 fish screen gave the following results: ♂ VTG↑, ♂ secondary sexual characteristics ↓, fecundity and fertility ↓. LOECs for effects on VTG in the range 0.6-37 µg/l, and for effects on secondary sexual characteristics in the range 0.6-113 µg/l. LOECs for reduced fecundity and fertility were 113 µg/l. (Biever *et al*. 2007). A non-standard version of TG 229 in which only ♂♂ were exposed to tert-OP gave a NOEC for ↑♂ VTG of 20 µg/l and for ↓ fecundity of <20 µg/l (Gronen *et al*., 1999).
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	TG 421- no endocrine or reproductive effects noted at oral doses up to 500 mg/kg/day.	TG 230 fish screen: ♂ VTG↑ (NOEC = 27.8 µg/l) (Japanese Ministry of the Environment 2006). In a non-standard assay, hepatic ♂ VTG ↑ (NOEC = 27.8 µg tert-OP/l) (Nozaka *et al*. 2004).
	AR antagonism: AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c.	Non-standard short-term studies with fish show ♂ and ♀ VTG ↑ (or ♂ VTG mRNA transcripts ↑; and/or ♂ ZPP/ZRP ↑) in a large range of species after

Larval Amphibian Growth and Development Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	either tert-OP injection, feeding or ambient exposure. Ambient water NOECs for these effects ranged from 1.6 to 10 µg/l. (Andreassen & Korsgaard 2000; Andreassen *et al*. 2005; Bangsgaard *et al*. 2006; Bjerregaard *et al*. 2008; Brian *et al*. 2005; Chikae *et al*. 2003; Genovese *et al*. 2011; Huang & Wang, 2001; Jespersen *et al*. 2010; Jobling *et al*. 1996; Karels *et al*. 2003; Knudsen *et al*. 1998; Madsen *et al*. 2002, 2003, 2006; Ortiz-Zarragoitia & Cajaraville 2005; Pait & Nelson 2003; Pedersen *et al*. 1999, 2003; Rasmussen & Korsgaard 2004; Routledge *et al*. 1998; Van den Belt *et al*. 2003; Zhang *et al*. 2010). Some of these studies also reported reductions in milt volume, GSI, smolting ability, growth and mating behaviour, or abnormalities in testicular histology and spermatocrit.
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is	Tert-OP was positive in both the immature and the ovariectomized rat assay	In a standard FSDT (OECD, 2011), the most sensitive fish

Larval Amphibian Growth and Development Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).	for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	species (zebrafish) gave NOECs of 9.5-17.6 µg/l for ♂ VTG induction and 5.7-<13.8 µg/l for ♀-biased sex ratio. A test with medaka (Knörr & Braunbeck 2002) gave a NOEC of 20 µg/l for ♀-biased sex ratio. A second test with medaka (Seki *et al*. 2003) gave a NOEC for ♀-biased sex ratio of 23.7 µg/l. In a non-standard test similar to the FSDT, Gray *et al*. (1999a) demonstrated a NOEC of <100 µg/l for testis-ova induction in medaka. However, Toft & Baatrup (2003) reported no effect on sex ratio in guppies at concentrations up to 200 µg/l, but the NOEC for adverse effects on sperm count and gonopodial length was 11.7 µg/l.
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	In partial lifecycle non-standard fish tests, results reported range from no effects on sexual development (Carlson *et al*. 2000), to reduced courtship behaviour, reduced fertilisation success, reduced overall reproductive success,

Larval Amphibian Growth and Development Assay data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			testicular abnormalities, lower GSI, reduced body colouration, reduced testosterone and increased estradiol. NOECs in ambient water for these effects range from <10 to 100 µg/l (Gray *et al*. 1999b; Kinnberg & Toft 2003; Mills *et al*. 2001; Toft & Baatrup 2001; Vázquez *et al*. 2009; Zaroogian *et al*. 2001).
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).	In a non-standard female PP assay VO was advanced by 3.2 days at oral doses of 200 mg/kg/day. No other parameters were determined.	Short term exposures of zebra finch chicks to orally dosed tert-OP (206 mg/kg body wt.) caused increases in oviduct weight (Millam *et al*. 2002).
	PR binding (weak affinity) (Laws *et al* 2000)		Partial lifecycle tests with birds produced results showing no impact on reproductive success or song control nuclei at 20-206 mg/kg body wt.(Millam *et al*. 2001; Quaglino *et al*. 2002).

Table 5.7.2.2: Conclusions about Larval Amphibian Growth and Development Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP

Conclusions for tert-OP	Next Step (as BB)	Assay result for tert-OP
Strong evidence for *in vivo* endocrine activity in amphibians and other species	Regulatory authorities may consider that further data from amphibians are not required.	n/a

Table 5.7.2.3: Other considerations and conclusions for octylphenol. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for octylphenol.

Other considerations (as given in BB)	Conclusions for tert-OP
The LAGDA does not cover the reproductive phase of the lifecycle, but a lifecycle test which could be used to address any concerns about reproduction is not currently available.	Despite some estrogenic responses (VTG induction in larvae; oviduct formation in a few males), no significant effects were observed on E2 or T, sex ratio or gamete counts. Only a lifecycle test could decide whether these relatively mild effects could be translated into impacts on reproductive success, and it seems doubtful whether such a test would be justified in this case.

62. Overall conclusions about Larval Amphibian Growth and Development Assay and existing data: There are overwhelming data from fish that tert-OP has estrogenic impacts on reproduction. The non-standard data for *X. tropicalis* (Porter *et al*., 2011), which have been taken as roughly equivalent to LAGDA data, show that tert-OP can also cause estrogenic effects in amphibians, and this is supported by non-standard data from several other amphibian species. However, there are no data on reproductive success, and the Porter *et al*. (2011) data do not indicate strongly that reproductive impacts are to be expected. Some regulatory authorities might feel that read-across from fish would justify a request for amphibian lifecycle data (despite the lack of a standardised protocol), although risk management for effects on fish populations would be likely also to protect amphibians given the similar sensitivity of their mechanistic estrogenic responses.

5.7.3 Avian two generation test (ATGT) (GD No. 150 Section Annex 2.4)

No data available

5.8 4-Tert-Octylphenol Case Study Results: *In vivo* Mammalian Assays That Have Not Yet Completed Validation Or Not Primarily Designed For Detection Of Endocrine Disruption

5.8.1 Adult male assay (GD No. 150 Section Annex 2.5)

No data available

5.8.2 TG 408: Repeated Dose 90 Day Oral Toxicity Study in Rodents (GD No. 150 Section Annex 2.6)

Scenario J : Result -, existing *in vitro* data +, existing *in vivo* data +

Scenario J is appropriate because tert-OP was negative in a standard TG 408 assay. It is acknowledged that some slight changes were reported in non-standard assays (see Column 1 Table

5.8.2.1) but the data were mostly negative and therefore for the purposes of this case study the negative assay result scenario has been considered. Existing *in vitro* data show a variety of responses including ER binding and agonism, AR binding and antagonism, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights in some TG 407 assays and positive results in UT assays are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.8.2.1: tert-OP data summary

Repeated Dose 90 Day Oral Rodent Toxicity Study data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
In a standard assay where tert-OP was administered up to 230 mg/kg/day, there were no effects on endocrine endpoints in male or female rats (Suberg *et al*, 1982). In a more recent non-standard assay male rats were administered OP by gavage (up to 125 mg/kg/day) for 60 days. Some minimal effects on caudal sperm counts were reported but there no effects on SAT weight or histopathology. There were also no effects on testicular gene expression (Gregory *et al* 2009).	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	Fish hepatocyte assay: VTG production was increased in a	TG 421- no endocrine or reproductive effects noted at oral doses up to 500	

Repeated Dose 90 Day Oral Rodent Toxicity Study data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	mg/kg/day.	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b; Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	

Repeated Dose 90 Day Oral Rodent Toxicity Study data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).		
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)	In a non-standard female PP assay VO was advanced by 3.2 days at oral doses of 200 mg/kg/day. No other parameters were determined.	

Table 5.8.2.2: Conclusions about Repeated Dose 90 Day Oral Rodent Toxicity Study and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay Result for tert-OP
No evidence that tert-OP has (anti)-EATS activity in TG 408. The existing *in vitro* an *in vivo* data indicate that activity is weak and therefore may not be detected by this assay.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system (level 2).	Tert-OP binds to ER and AR and is an agonist in the ER STTA assay and an antagonist in the AR STTA assay. Conduct of these assays with additional metabolizing systems is therefore

Conclusions for tert-OP	Next Step (as BB)	Assay Result for tert-OP
In addition, effects that were detected in the TG 407 assays, used higher oral doses than were employed in TG 408 (400 mg/kg/day and above comnpared to up to 230 mg/kg/day.		unnecessary. It is likely that dosing issues and/or the metabolism of tert-OP *in vivo* is the reasons for the negative result in the TG 408 assay. ADME studies have indicated that tert-OP undergoes rapid first pass metabolism.
	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.

Table 5.8.2.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for tert-OP.

Other considerations (as given in BB)	Conclusions for tert-OP
If existing data are from an adequate level 5 assay then question why differences.	The "mammalian effects of concern" in this case are provided by the positive UT assays for ER agonism (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a) and by some effects in TG 407 studies (Bian *et al* 2006; Hossaini *et al* 2003; Laws *et al* 2000). On the other hand, H assays and some TG 407 studies are negative CIPT, 1994; HRC, 1994; Sahambi *et al*, 2010). The lack of effects in the 2-gen assay (TG 416) (Tyl *et al* 1999) indicates that although the tert-OP is an estrogen agonist *in vivo* in the UT assay, it does not have adverse effects on endocrine endpoints, reproduction or development in intact animals when administered at doses up to 111-369 (dependent on the stage of the study). The positive results in TG 407 assays were generally obtained at doses above this. The data where s.c. administration was used did not give consistent results. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it

Other considerations (as given in BB)	Conclusions for tert-OP
	includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention).
Effects seen in existing studies may be in a more sensitive life stage.	This is unlikely to be the case as the 2-gen (TG 416) study was negative.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Higher doses were used in TG 407 assays where reductions in SATs were observed (Bian *et al*, 2006; Hossaini *et al* 2003) compared to in the TG 408 assays. This may explain the different results. tert-OP also undergoes rapid first pass metabolism when given orally and this may also account for differences between results using oral and s.c. dosing.Higher internal doses may be achieved when tert-OP is adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
Further mechanistic studies would help determine MoA.	Valid consideration although many mechanistic studies already exist.

63. Overall conclusions about Repeated Dose 90 Day Oral Rodent Toxicity Study and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence that tert-OP can interact with endocrine systems although data from the 90 day oral toxicity assay indicate that any endocrine activity does not result in effects on endocrine-sensitive endpoints when administration is via the oral route. ER agonism was, however, demonstrated *in vivo* in the UT assay. It is possible, therefore that administration of tert-OP by other routes, where a higher internal dose may be achieved, may give different results. In mammalian species, the available evidence from a level 5 test also indicates that this interaction does not result in effects on reproduction when test-OP is administered via oral routes. Interestingly,, in fish and amphibians, adverse effects on sexual development and/or reproduction do occur, probably attributable to ER binding.

5.8.3 OECD TG 451-3: Combined Chronic Toxicity/Carcinogenicity Studies (GD No. 150 Section Annex 2.7)

No data available

5.8.4 OECD TG 421 Reproduction/Developmental Toxicity Screening Test and TG 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (GD No. 150 Section Annex 2.8)

Scenario J : Result -, existing *in vitro* data +, existing *in vivo* data + Scenario J is appropriate because tert-OP was considered to be negative in standard and non-standard TG 421 assays. Effects on endocrine-related endpoints were only reported at doses causing severe systemic toxicity (see Column 1 Table 5.8.4.1) and therefore for the purposes of

this case study the negative assay result scenario has been considered. Existing *in vitro* data show a variety of responses including ER binding and agonism, AR binding and antagonism, steroidogenesis disruption and possibly TH antagonism. Existing *in vivo* data have some positive and some negative results. The positive results such as estrus cycle disturbance and reduced SAT weights in some TG 407 assays and positive results in UT assays are consistent with effects on E and A modalities therefore the existing *in vivo* data were deemed to be positive for the purposes of this scenario.

Table 5.8.4.1: tert-OP data summary

Reproduction/Developmental Toxicity Screening and Combined Repeated Dose Toxicity Study data for tert-OP	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
In a standard assay tert-OP was administered orally at 125, 250 or 500 mg/kg/day. Severe systemic toxicity occurred at 500 mg/kg with impaired reproduction and decreased weight of SATs. At the lower doses there were no effects on endocrine-related endpoints, reproduction or development (HRC 1995). In a non-standard assay using few animals (4/group) but with additional endocrine endpoints rats were administered tert-OP by gavage at 100 mg/kg/day). There were no effects on endocrine-related endpoints (including hormone levels), reproduction or development (Piesma *et al* 1998a,b).	ER binding to mammalian and wildlife isoforms. (Laws *et al*, 2000, 2006; Strunck *et al*, 2000; Yoon *et al*, 2000; Olsen *et al* , 2005; Routledge *et al*, 2000; Sahambi *et al*, 2010; Andreassen & Korsgaard 2000; Denny *et al*. 2005; Lutz & Kloas 1999)	TG 407 - reduced SAT weights in rats (♂) at 450 mg/kg/day (Bian *et al*, 2006) and 400 mg/kg/day (Hossaini *et al* 2003), disurbance of estrus cycle at 200 mg/kg/day (Laws *et al* 2000). No endocrine-related effects observed at ≤ 300 mg/kg/day (CIPT, 1994), at ≤ 250 mg/kg/day (HRC, 1994) or ≤ 125 mg/kg/day (Sahambi *et al*, 2010).	No data
	ER agonism : human ER STTA. (OECD 2006a; Yamasaki *et al* 2002; Ghisari *et al* 2009; Wu and Safe 2007; Isidori *et al* 2006). ER agonist reponse in MCF7 cell proliferation assays. (Rajapakse *et al*. 2004; Olsen *et al* 2005; Sahambi *et al* 2010). ER agonism in transactivation assays using ER derived from fish. (Fu *et al*. 2007; Gale *et al*. 2004)	TG 408- no endocrine-related effects noted at ≤ 230 mg/kg/day (Suberg *et al*, 1982) or ≤ 125 mg/kg/day (Gregory *et al* 2009).	

Reproduction/Developmental Toxicity Screening and Combined Repeated Dose Toxicity Study data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Fish hepatocyte assay: VTG production was increased in a number of fish species. (Jobling & Sumpter 1993;Monteverdi & Di Giulio 1999; Olsen *et al*. 2005; Petersen & Tollefsen 2011; Park *et al*. 2003; Segner *et al*. 2003; Tollefsen *et al*. 2003; Toomey *et al* 1999; White *et al*. 1994)	TG 416- no endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). A smaller study using s.c. exposure at 100 mg/kg/day showed reduction in some SAT weights (♂) and some hormone changes in males (Yoshida *et al*, 2001).	
	AR binding. (Paris *et al* 2002; Li *et al* 2010).	Pre/postnatal dosing (up to 250 mg/kg) by oral gavage had no effect on fetal malformations (Harazono *et al* 2001). Studies using s.c. administration did not give consistent results. Treatment of 4 week old rats with 20-80 mg/kg/day for 1 month resulted in reduced SAT weight (♂) (Kim *et al*, 2004). Treatment during pregancy or in the neo-natal period in some studies (up to 200 mg/kg) had few or no endocrine-related effects (Haavisto *et al* 2003; Aydogan and Barlas 2006; Götekin and Barlas 2008; Sonne-Hansen *et al* 2003; Mikkilä *et al* 2006; Williams *et al* 2001a, b;	

Reproduction/Developmental Toxicity Screening and Combined Repeated Dose Toxicity Study data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		Sharpe *et al* 2003). However, treatment of newborn female pups with 100 or 50 mg/kg/day s.c. caused changes such as premature vaginal opening or persistent estrus (Willoughby *et al* 2005; Katsuda *et al* 2000b).	
	AR antagonism :AR STTA. (Krüger *et al* 2008; Paris *et al* 2002).	Tert-OP was positive in both the immature and the ovariectomized rat assay for agonism at oral doses of 100 mg/kg/day or s.c. doses of 50 mg/kg/day and above (Laws *et al* 2000; Diel *et al* 2000; Kwack *et al* 2002; Yamasaki *et al* 2002; Katsuda *et al* 2000a).	
	Steroidogenesis disruption: In non-standard assays using rat Leydig cells there is evidence for effects on testosterone and progesterone. (Murono *et al* 2000, 2001, 2002; Haavisto *et al* 2003). In the H295R assay, cortisol was reduced and some some enzymes inhibited (Nakajiin *et al* 2001).		
	Effects in thyroid assays. Stimulation of cell growth and inhibition of T3-stimulated cell growth (Ghisari *et al* 2009).	Tert-OP was negative in the H castrated rat assay for agonism and antagonism at oral doses of 50-200 mg/kg/day (Yamasaki *et al* 2003).	
	Some effects on hormone secretion from isolated rat ovarian follicles. (Myllymaki *et al*, 2005).		
	PR binding (weak affinity) (Laws *et al* 2000)	In a non-standard female PP assay VO was advanced by 3.2 days at oral doses of 200 mg/kg/day. No	

Reproduction/Developmental Toxicity Screening and Combined Repeated Dose Toxicity Study data for tert-OP	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		other parameters were determined.	

Table 5.8.4.2: Conclusions about Reproduction/Developmental Toxicity Screening and Combined Repeated Dose Toxicity Study and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with tert-OP for the suggested assay is shown if available.

Conclusions for tert-OP	Next Step (as BB)	Assay Result for tert-OP
No evidence that tert-OP has adverse effects in TG 421. The existing *in vitro* an *in vivo* data indicate that any endocrine activity is weak and therefore may not be detected by this assay. In addition, the dose levels used in the TG 421 assays were either too high and resulted in systemic toxicity (500 mg/kg) or were lower than those the doses where effects were detected in the TG 407 assays. No effects were observed in TG 421 at 250 mg/kg/day and below whilst decreased SAT weight was observed in TG 407 at 400 mg/kg/day and above.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system (level2).	Tert-OP binds to ER and AR and is an agonist in the ER STTA assay and an antagonist in the AR STTA assay. Conduct of these assays with additional metabolizing systems is therefore unnecessary. It is likely that dosing issues and/or the metabolism of tert-OP *in vivo* is the reasons for the negative result in the TG 421 assay. ADME studies have indicated that tert-OP undergoes rapid first pass metabolism.
	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	2-Gen (TG 416): negative. No endocrine or reproductive effects noted at oral doses up to 111-369 mg/kg/day. Study considered to be robust and included measurement of sensitive endocrine endpoints (Tyl *et al*, 1999). However it is noted that this study does lack some important endocrine endpoints.

Table 5.8.4.3: Other considerations and conclusions for tert-OP. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for ter-OP.

Other considerations (as given in BB)	Conclusions for tert-OP
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Higher doses were used in TG 407 assays where reductions in SATs were observed (Bian *et al*, 2006; Hossaini *et al* 2003) compared to those used in the TG 421 assays. This may explain the different results. Very high doses (500 mg/kg and above) caused severe systemic toxicity in TG 421. tert-OP also undergoes rapid first pass metabolism when given orally and this may also account for differences between results using oral and s.c. dosing.Higher internal doses may be achieved when tert-OP is adminstered by the s.c. route. Oral dosing is however, more realistic for human exposure.
Effects seen in existing studies may be in a more sensitive life stage.	This is unlikely to be the case as the 2-gen (TG 416) study was negative.
Further mechanistic studies would help determine MoA.	Valid consideration although many mechanistic studies already exist.

64. Overall conclusions about Reproduction/Developmental Toxicity Screening and Combined Repeated Dose Toxicity Study and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence that tert-OP can interact with endocrine systems although the available evidence from the Reproduction/Developmental Toxicity Screening and Combined Repeated Dose Toxicity Study indicates that any endocrine activity does not result in effects on reproduction when administration is via the oral route. ER agonism was, however, demonstrated *in vivo* in the UT assay. It is possible, therefore that administration of tert-OP by other routes, where a higher internal dose may be achieved, may give different results. T he available evidence from a level 5 test also indicates that this interaction does not result in effects on reproduction when test-OP is administered via oral routes. Interestingly, in fish and amphibians, adverse effects on sexual development and/or reproduction do occur, probably attributable to ER binding.

5.9 4-Tert-Octylphenol Case Study: Conclusions

5.9.1 *In vitro* Assays

65. Tert-OP data exist for all of the *in vitro* tests under consideration with the exception of the aromatase assay. All of the assays were positive although the "*in vivo*" effects of concern" were a mixture of studies where effects on endocrine endpoints had been reported and where no effects on endocrine endpoints were reported. Taken as a whole, the presence of positive results meant that the existing positive *in vitro* data were combined with positive and *in vivo* data of concern so that Scenario A was considered in all cases. However, the decision to consider the "*in vivo*" effects of concern" as positive was not taken on a weight of evidence basis, which was outside the scope of the case study. The effects in ER- and AR-based assays

(binding and transactivation assays were based on standardized tests and were therefore considered to be largely reliable. The positive response in the steroidogenesis assay should, however,be treated with caution as non-standard assays using rat Leydig cells were employed. This system was not considered to be reliable when it underwent validation (US EPA, 2006).

66. The assay results provided useful information for both mammals and wildlife and the "next steps" as given in the building blocks appeared logical and useful when tested with the tert-OP data. In all cases it was considered that further *in vivo* testing was not justified as sufficient data, at all levels of the CF, were already available for both mammals and wildlife. The "other considerations" were also very useful, for example, consideration of the charactertistics of metabolites of the chemical. In the case of tert-OP, its glucuronide metabolite had been tested in ER and AR transactivation assays and shown to be negative.

5.9.2 *In vivo* Wildlife Assays

67. The dataset for fish (both standard and non-standard) is reasonably complete, but standard data for amphibians and birds are lacking although some non-standard data for these groups have been found. The non-standard fish data largely support the observations made in the standard fish screens and tests *i.e.* that tert-OP is estrogenic in many fish species and can have deleterious effects on reproduction. There is limited read-across from amphibians and birds which supports the conclusion of *in vivo* estrogenicity. Other modes of action (*e.g.* interference with steroidogenesis) may be occurring in fish, but there is no firm *in vivo* evidence for this MOA.

68. The building blocks providing advice on the wildlife assays for which data exist generally produce logical outcomes when compared with the results of higher level tests, and especially when one considers data from the fish full lifecycle tests (FFLCT). Data are not available from an MMGT, but tert-OP is not expected to cause effects at lower concentrations in that test compared with the FFLCT. There are no instances of a lower level wildlife assay giving a false negative when compared with higher level wildlife data, neither are there any false positives. The TG 229 and 230 *in vivo* fish screens appear to be of broadly similar sensitivity to tert-OP, and indeed are of similar or occasionally greater sensitivity compared with the FSDT (TG 234) and the FFLCT. Generally speaking, the lowest NOECs at all fish testing levels are of comparable magnitude, although some fish species appear to be of lower sensitivity than the OECD standard species.

69. As very few reproductive data are available for amphibians and birds, it is impossible to decide whether the fish data can be extrapolated to these groups. However, the abundant evidence for the estrogenicity and apical reproductive impacts of tert-OP in mammals and fish, and the limited supporting data for estrogenicity in amphibians and birds, suggest that tert-OP may be able to cause some apical reproductive effects in the latter groups.

5.9.3 *In vivo* Mammalian Assays

70. There are data available for tert- OP for most of the mammalian assays considered in the GD. The exceptions are the, male and female PP assays, extended 1 generation assay (TG 443), the adult male assay and and TG 453/452 (Combined chronic toxicity/ carcinogenicity studies). The outcome of the assays were a mixture of negative and positive results in the presence of positive "Mechanism (*in vitro* mechanistic data)" and "Mammalian Effects (*in vivo* effects of concern)" allowing the testing of scenarios A and J in this case study. The assay sensitive to ER-related modalities (UT assay) was unequivocally positive, supporting this mode of action being the primary endocrine effect of tert-OP. The H assay was negative, indicating that the interaction with AR observed *in vitro* did not translate into effects *in vivo*. Of the remaining assays tested, only non-standard TG 407 assays were positive at dose levels exceeding those given in the standard tests. The validation studies on TG 407 indicated that this assay is only sensitive to

strong estrogens such as ethinylestradiol and tert-OP does not fall into this category, supporting the results seen here. The remaining assays (TG 408, TG 421/422 and TG 415) were all negative when tert-OP was administered orally. It is noted that a number of studies have shown effects following administration by non-oral routes but the data were of variable quality and results were conflicting so they were not analysed in depth here.

71. In all cases, the advice provided by the GD was logical and helpful, suggesting relevant next steps for testing and providing suggestions as to why negative results may have been obtained. For example, consideration of ADME characteristics is highly relevant for tert-OP when comparing results obtained via different routes. It is noted that current TGs do not cover potentially relevant types of adverse effects which may be caused by oestrogenic chemicals such as e.g. mammary gland development, time for onset of puberty and breast cancer development. In no cases was it thought necessary to actually conduct a new test as an adequate 2-generation assay already existed. However, it was also noted that this assay did not include as many endocrine-sensitive endpoints as are included in TG 443 (ext-1-gen assay) and therefore this assay would provide a more comprehensive assessment

5.9.4 Overall Conclusions About Tert-Octylphenol

72. In summary, although data on tert-OP from a number of assays are unavailable, this case study suggests that the evaluated building blocks generally provide sound advice about data interpretation and possible next steps. It also seems likely that more restricted 'Existing Data' would not lead to substantially different conclusions, although this was not formally evaluated.

6. PERCHLORATE CASE STUDY RESULTS

6. 1 Data used in the Perchlorate Case Study Analysis

73. Data for standardised assays used in the case study on perchlorate for GD No. 150 are given in Table 6.1.1. The format of the table follows that given in the Contents of the GD so that the two documents can be easily compared. Data, from non-standardised assays, considered relevant for assessment of endocrine effects are given in Table 6.1.2. These data do not comply with the Test Guideline study designs in the CF but are considered to be of relevance for assessment of effects on the health of humans or wildlife.

Table 6.1.1: Data for standardised assays used in the case study on perchlorate for The OECD GD on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (No. GD 150). Note that the "Result" column indicates a positive (+), negative (-) or equivocal (Eq) result for endocrine endpoints only.

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
	A. Validated assays for which guidance is provided in the main GD			
	***In vitro* Screens**			
C.2.1	ER Binding Assay (US EPA OPPTS 890.1250)		No data	
C.2.2	AR Binding Assay (US EPA OPPTS 890.1150)		No data	
C.2.3	OECD TG 455: The Stably Transfected Human ERα Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA) (including Guidance for the Antagonism Assay)		No data	
C.2.4	OECD TG 456: H295R Steroidogenesis Assay		No data	
C.2.5	Aromatase Assay (US EPA OPPTS 890.1200)		No data	
	Wildlife Screens and Tests			
C.3.1	OECD TG 229: Fish Short Term Reproduction Assay (FSTRA)	-	**Standard assay:** Fathead minnow (*Pimephales promelas*) were exposed to perchlorate at measured 5.6-44 mg/l for 21 d. There were no effects on **fecundity**,	USEPA 2006

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
			hatching, **male histology** or **VTG**.	
		Eq	There was an increase in the numbers of **atretic ovarian follicles** and a decrease in the number of **stage 1A oocytes** (LOEC = 5.6 mg/l), but it is unclear whether this effect was related to endocrine disruption.	
			Non-standard assay:	
		+ + +	Adult zebrafish (*Danio rerio*) were exposed to measured perchlorate concentrations of 18 and 677 mg/l for up to 8 weeks. Both concentrations caused **thyroidal angiogenesis**, and the lower concentration also caused **thyroidal hyperplasia** and **colloid depletion**. However, reproductive performance (**fecundity**) was only depressed (by ~85%) at the higher concentration (NOEC = 18 mg/l).	Patiño *et al*. 2003
C.3.2	OECD TG 230: 21 Day Fish Assay		**Standard assay:** No data **Non-standard assay:**	
C.3.3	OECD GD 140: Variant of OECD TG 230: Androgenised Female Stickleback Screen (AFSS)		No data	
C.3.4	OECD TG 234: Fish Sexual Development Test (FSDT)		**Standard assay:** No data **Non-standard assay:**	
		+	Larval zebrafish (*Danio rerio*) were exposed from 3 d post-fertilisation for 30 d to nominal Perchlorate concentrations of 100 or 250 mg/l. Both treatments caused **hypothyroidism** which could be reversed by co-treatment with exogenous T4. Perchlorate also	Mukhi *et al*. 2007

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
		+	suppressed **growth** (body length) (by ~15% at the LOEC) and	
		+	skewed the **sex ratio** towards females (from 48% in controls, to 58% at the LOEC, and to 65% at the top concentration), an effect which was also counteracted by exogenous T4. The NOEC for these effects was < 100 mg/l.	
C.3.5	Fish Lifecycle Toxicity Test (FLCTT) (USEPA OPPTS 850.1500)		**Standard assay:** Post-fertilisation embryos (F0) of medaka (*Oryzias latipes*) were exposed to perchlorate to adulthood, and their offspring (F1) exposed to 60 d post-fertilisation. Mean measured concentrations of perchlorate were 66, 133, 273, 545 and 1090 µg/l.	ABC (2009)
		-	There were no significant changes in **any apical endpoint,** including reproduction and growth, at any concentration in either the F0 or F1 generations (NOEC > 1090 µg/l).	
		+	However, female **thyroid follicular hypertrophy** (prevalence: 3 out of	
		+	12 fish) and **thyroid colloid depletion** (6 out of 14 fish) occurred, with an overall NOEC of 273 µg/l in the F0 fish. The overall NOEC was 545 µg/l in the F1 fish.	
			Non-Standard assay:	Bernhardt & von Hippel 2008
		-	Three-spined stickleback (*Gasterosteus aculeatus*) were exposed for up to 1 year, through the stage of reproductive activity, to measured concentrations of perchlorate ranging from 30 to 100 mg/l. There were no observed changes in **reproductive behaviour** or **reproductive output** of adults exposed for 22 d to concentrations up to 18.6 mg/l.	
		+	However, in fish exposed throughout development, **swimming performance**,	

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
			reproductive behaviour, **survivorship** and **recruitment** were affected at all concentrations (NOEC < 30 mg/l), and only 25% of males produced fry at the LOEC (30 mg/l). Reproductive activity was almost completely inhibited at 100 mg/l, and no males produced fry at this concentration. It seems likely that these effects are a form of endocrine disruption (see Bernhardt *et al*. 2006), but the mechanism was not elucidated.	
C.3.6	OECD TG 231: Amphibian Metamorphosis Assay (AMA)		**Standard assay:** Nominal perchlorate concentrations tested against clawed frog *Xenopus laevis* larvae for 21 d were 0, 62.5, 125, 250, and 500 µg/l, and measured concentrations were generally within 20% of these.	OECD 2007a
		+	**Whole-body length**, **snout-vent length** and **wet wt**. all increased in *X. laevis* larvae (by about 5-20%) in response to perchlorate, with NOEC values generally in the range 62.5-125 µg/l. Hind limb length decreased by 20-30% (NOEC = 125 µg/l).	
		+	**Thyroid histology** was generally altered at all concentrations (NOEC <62.5-62.5 µg/l), including increases in thyroid epithelial cell height and thyroid volume/area.	
			Non-Standard Assay: Clawed frog (*Xenopus laevis*) eggs and larvae were exposed to measured perchlorate concentrations between 5 and	Goleman *et al*. 2002b
		+	425,000 µg/l for 70 d. **Forelimb emergence** was reduced (by ~50% at the LOEC) at all concentrations (NOEC < 5 µg/l), while NOECs for	
		+	reduced **hindlimb length** and reduced **tail resorption** were both 5 µg/l. In a USEPA standard screening test, in which tail	

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
		+	resorption in stage 60 larvae was measured, 19,800 µg perchlorate /l exposure for 14 d reduced **tail resorption** from 96 to 17%.	Opitz *et al*. 2009
		+	Clawed frog (*Xenopus laevis*) larvae were exposed to a measured concentration of perchlorate at 20 mg/l for 12 d. This treatment **delayed development**, and **hind limb length** was reduced by ~50%.	
		+	These changes were accompanied by **thyroid histopathology**, reduced expression of genes regulated by thyroid hormone, and up-regulation of thyroid-stimulating hormone tshb-A mRNA.	Tietge *et al*. 2005
		+	Clawed frog larvae (*Xenopus laevis*) at Gosner stage 51-54 were exposed to measured perchlorate at 16, 63, 250, 1000 and 4000 µg/l for 14 d. **Metamorphosis** was significantly retarded (NOEC = 63 µg/l), but **histological effects** were observed at the lowest concentration (NOEC < 16 µg/l). A similar experiment with stage 51 larvae exposed to 8, 16, 32, 63 and 125 µg/l gave a NOEC for **retarded metamorphosis** of 63 µg/l, and a NOEC for **thyroid hypertrophy** of 32 µg/l.	
C.3.7	OECD TG 206: Avian Reproduction Test	+	**Standard Assay:** In this standard assay with Japanese quail (*Coturnix japonica*), the birds were orally dosed with perchlorate at concentrations of 100, 250, 500 and 1000 mg/kg feed (corresponding to 13.8, 36.4, 64.7 and 144.1 mg/kg body wt./day). There were no significant effects on most endpoints, but **eggshell thickness** was reduced to 91% of the control value at the LOEC (NOEC = 100 mg/kg feed), and	Springborn Laboratories, 2011

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
		+	**male weight gain** was reduced to 76% of the control value at the LOEC (NOEC = 500 mg/kg feed).	
			Non-Standard Assay:	Chen *et al*. 2008
			Laying female Japanese quail (*Coturnix japonica*) were exposed to perchlorate at 2000 or 4000 mg/l in drinking water. Thyroid status	
		+	was examined in the resulting embryos. **Hypothyroidism** was observed in the adults of both	
		+	treatment groups (NOEC < 2000 mg/l), and **egg production** was decreased in the high dosage group only (NOEC = 2000 mg/l). The	
		+	embryos from both groups also	
		+	experienced **hypothyroidism**, as	
		+	evidenced by **thyroid gland hypertrophy** and lower **thyroid hormone storage**. This was	
		+	associated with decreased	
		+	**embryonic growth**, delayed **hatching** and increased **mortality** during hatching.	
				Gentles *et al*. (2005)
			Adult female northern bobwhite quail (*Colinus virginianus*) were exposed to perchlorate at drinking	
		-	water 1.2, 11.7 and 117 mg/l for 30	
		-	d. These doses did not affect **body or organ weights**, or **egg**	
		+	**production**, but the top dose caused alterations of **thyroid gland morphology** (NOEC = 11.7 mg/l).	
		+	These changes included ~50%	
		+	reduction in **colloid area** and ~30% increase in **follicle cell height**.	
	Mammalian Screens and Tests			
C.4.1	OECD TG 440: Uterotrophic Bioassay in Rodents (UT assay) (Including GD on the Use of the Assay to Screen for Anti-Estrogenicity)		No data	
C.4.2	OECD TG 441:		No data	

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
	Hershberger Bioassay in Rats (H Assay) (Including OECD GD for Weanling Hershberger Bioassay)			
C.4.3	Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats (Male PP Assay) (US EPA OPPTS 890.1500)	+	**Standard Assay:** Ammonium perchlorate was administered by gavage at 62.5, 125, 250, and 500 mg/kg/day to male Wistar rats (n=15) from PND 23 to 53. T4 was decreased in a dose-dependent manner from 125 mg/kg/day, TSH was increased at the same doses, whilst T3 was unaffected. Thyroid histology was significantly altered at all doses with a clear dose-dependent decrease in colloid area and increase in follicular cell height. No effects on preputial separation or reproductive tissues weight were observed.	Stoker *et al* (2006)
C.4.4	Pubertal Development and Thyroid Function Assay in Peripubertal Female Rats (Female PP Assay) (US EPA OPPTS 890.1450)	+	**Standard Assay:** Ammonium perchlorate was administered by gavage at 62.5, 125, 250, and 500 mg/kg to female Wistar rats (n=15) from PND 22 to 43. T3 and T4 were decreased in a dose-dependent manner from 125 mg/kg/day, TSH was increased at 250 and 500 mg/kg. Thyroid histology was significantly altered at all doses with a dose-dependent decrease in colloid area and increase in follicular cell height. No effects on vaginal opening or weights of uterus and ovaries were observed. There were no treatment-related effects on estrous cyclicity.	US EPA (2007) Laws (2011)
C.4.5	OECD TG 407: Repeated Dose 28 Day Oral Toxicity Study in Rodents		No data	
C.4.6	OECD TG 416: Two-Generation Reproduction Toxicity Study (Including TG 415: One-Generation		**Standard Assay:** Male and female rats (n=30) were exposed to ammonium perchlorate in drinking water at 0, 0.3, 3 and 30	Argus (1999, reported in NAS, 2005

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
	Reproduction Toxicity Study)		mg/kg/day. Males and females were allowed to cohabitate for 14 days. Parental (P) rats and F1 pups not selected for breeding were killed on PND 21. F1 pups selected for breeding were dosed through post weaning, cohabitation and lactation periods. After birth of the F2 generation, all F1 parents and F2 pups were killed on PND 21. There were no effects on reproduction or gestation length and no deaths or abortions. There were no effects on mating, fertility, sperm parameters or estrus cyclicity in either P or F1 generations. There were no changes in numbers of live pups, viability, sex ratios or pup body weights. Endpoints of sexual maturity (*e.g.* VO, PPS) or AGD were not determined.	and CEPA, 2004); York *et al* (2001).
		+	Dose-dependent changes in thyroid weight and histopathology and hormone levels were observed in all generations. Relative thyroid weights were increased at 30 mg/kg/day (♀) and 3 & 30 mg/kg/day (♂) in the P generation, whilst in the F1 generation thyroid weights were increased at all doses (♀) and 3 & 30 mg/kg/day (♂). Hypertrophy and hyperplasia of the thyroid follicular epithelium increased in incidence and severity in a dose related manner. Reduced T4 & T3 and increased TSH also occurred in a dose-related manner but were not consistent across the generations and sexes. Two male rats from the F1 30 mg/kg/day group developed thyroid adenomas, compared to none in controls. These animals had had 19 weeks of dosing.	
C.4.7	OECD TG 443: Extended		No data	

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
	One-Generation Reproductive Toxicity Study			
	B. Assays that have not yet completed validation, or have not primarily been designed for detection of endocrine disruption, for which limited guidance is given in Annex 2 of the GD			
	***In vitro* Screens**			
Annex 2.1	The Human AR Transcriptional Activation Assay for Detection of Androgen (Ant)agonist-Activity of Chemicals (AR STTA)		No data	
	Wildlife Screens and Tests			
Annex 2.2	Fish (Medaka) Multi-Generation Test (MMGT)		No data	
Annex 2.3	Larval Amphibian Growth and Development Assay (LAGDA)	- +	**Standard assay** No data **Non-standard assay** Note that this test was performed with *Xenopus tropicalis*, although the LAGDA is now being developed using *X.laevis* and some different endpoints. These data cannot therefore be used to evaluate the advice pertaining to the LAGDA. *X. tropicalis* larvae (<48 h post-hatch) were exposed for up to 40 weeks to measured perchlorate concentrations of 56, 167, 500 and 1500 µg/l. None of these concentrations caused significant effects on metamorphosis, body size of adults, or gonadosomatic indices of adults. However, some thyroid histopathology was observed	Olmstead (2009)

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
			(NOEC = 170 µg/l), and vitellogenin levels in females were increased by a factor of approximately 1.7 (NOEC = 56 µg/l).	
Annex 2.4	Avian Two Generation Test (ATGT)		No data	
	Mammalian Screens and Tests			
Annex 2.5	Adult Male Assay		No data	
Annex 2.6	TG 408: Repeated Dose 90 Day Oral Toxicity Study in Rodents	+	**Non-Standard Assay:** Ammonium perchlorate was administered to rats (n=10) via drinking water at levels of 0.01, 0.05, 0.2, 1.0, and 10.0 mg/kg/day for 90 days. The study design included a non-treatment recovery period of 30 days to evaluate reversibility. The study also investigated potential effects on sperm parameters, estrous cyclicity and serum hormone levels (T3, T4 and TSH). No toxicologically significant differences were observed between the control and treated groups with respect to survival, clinical observations, body weights, food consumption, water consumption, haematology, clinical chemistry, estrous cycling or sperm parameters. In males and females at 10 mg/kg/day thyroid weights were increased and thyroid histopathology consisting primarily of follicular cell hypertrophy with microfollicle formation and colloid depletion was noted. These changes were reversible after a non-treatment recovery period of 30 days. Changes in TSH and thyroid hormones were observed at all dose levels; however, no thyroid organ weight or histopathological effects were observed at perchlorate dose levels ≤ 1.0 mg/kg/day.	Springborn *et al* (1998, reported in NAS, 2005 and CEPA, 2004); Siglin *et al* (2000)
Annex	OECD TG 453: Combined		**Non- Standard Assay:**	

GD Section	Tests and Screens	Result (+ - Eq)	Data for perchlorate	Reference
2.7	Chronic Toxicity/Carcinogenicity Studies (including TG 452 Chronic Toxicity Studies)	+	Male Wistar rats were administered sodium perchlorate (1%) via drinking water providing a dose of approximately 1,300 mg/kg/day. Rats were killed after 40, 120, 220 and 730 days of exposure. Body weights of control and treated animals were similar throughout. Thyroid weights of treated rats increased markedly compared to control rats at all time points. After 40 days, the treated rats developed follicular cell hyperplasia, colloid resorption and low-grade mesenchymal reaction. After 200 days, diffuse degenerative changes with fibrosis and increased colloid were observed. After 2 years, 4 of 11 treated rats developed benign thyroid tumours whilst 20 untreated controls had no thyroid tumours. BALBc mice were administered sodium perchlorate (1.2%) in drinking water providing a dose of approximately 2,100 mg/kg/day. Animals were killed after 46 weeks because of treatment-related deaths. Perchlorate treatment caused thyroid epithelial hypertrophy and hyperplasia. An increased incidence of thyroid follicular carcinomas was seen: controls 0/22, perchlorate treated 5/6)	Kessler and Kruskemper (1966, reported in NAS, 2005 and CEPA, 2004). Pajer and Kalisnik (1991, reported in NAS, 2005 and CEPA, 2004).
Annex 2.8	OECD TG 421 Reproduction/Developmental Toxicity Screening Test and TG 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test		No data	

Table 6.1.2: Data for non-standardised assays used in the case study on perchlorate

Summary of Non-Standard Perchlorate Data	**Reference**
Mechanism (*in vitro* mechanistic data)	
Endostyle tissue from larval lampreys (*Petromyzon marinus*) was incubated for 4 h with perchlorate. This resulted in a reduction of iodide uptake and reduced incorporation of iodine into lamprey thyroglobulin., leading to a reduction in total thyroglobulin.	Manzon & Youson (2002) [paper not seen]
The ability of substances (including perchlorate) to inhibit NIS-mediated iodide uptake *in vitro* was investigated using a CHO cell line stably expressing hNIS. Perchlorate ion was able to inhibit iodide uptake in a dose-dependent manner.	Agretti *et al* (2011)
Thyroid gland explant cultures from prometamorphic *Xenopus laevis* tadpoles were exposed to graded concentrations of perchlorate for 12 days and effects on T4 release determined. Perchlorate was a potent inhibitor of T4 release, IC(50) 13 ± 4.0 μM.	Hornung *et al* (2010)
The properties of the human NIS, stably transfected in COS-7 cells, were compared to those of a thyroid cell line, the FRTL5 cells, by measuring NIS-mediated transport directly. ^{125}I was transported in both systems. Transport was inhibited by competing anions and in both cell types the order of inhibitory magnitude of response, reflecting the affinity of the transporter, was $ClO_4^- > ReO_4^- > I^- > SCN^- > Br^-$.	Van Sande *et al* (2003).
Effects (*in vivo* effects of concern) **<u>Studies in wildlife species : Fish</u>**	
Three-spined stickleback (*Gasterosteus aculeatus*) were exposed to perchlorate at measured concentrations of 30 or 100 mg/l for almost a whole life cycle, from F0 adulthood to F1 reproductive maturity. Male nuptial colouration, courtship behaviour, and normal sexual development were depressed at both concentrations (NOEC < 30 mg/l), and genetic testing showed that some females were masculinised to the point where they possessed ovotestes and produced both types of gamete. Crosses produced using sperm from these fish failed to develop. The authors claim that these effects were an androgenic phenomenon.	Bernhardt *et al*. 2006
Adult mosquitofish (*Gambusia holbrooki*) were exposed to measured perchlorate concentrations of 0.1, 1, 10, 100 and 1000 mg/l for up to 30 d. Thyroid follicular epithelial cell height, hyperplasia and hypertrophy all increased (by up to 20% at the LOEC) with increasing perchlorate at 30 d, with a LOEC of 0.1 mg/l (NOEC < 0.1 mg/l), and whole body T4 was depressed by approx. 40% at 0.1 mg/l after 30 d.	Bradford *et al*. 2005
This review concludes that no firm links have been established between perchlorate	Carr & Patiño

(and other chemical)–induced alterations in the hypothalamus-pituitary-gonadal (HPT) axis and impacts on amphibian or fish populations, but that perchlorate is the only chemical for which a direct link has been established between HPT disruption and adverse effects on fish and amphibian development or reproduction in the laboratory.	2011
Fathead minnow (*Pimephales promelas*) were exposed from fertilisation to 28 d to nominal concentrations of perchlorate at 1, 10 or 100 mg/l. Thyroid hyperplasia and epithelial cell height increased by up to 30% at the LOEC in all groups (NOEC < 1 mg/l), and whole-body T4 increased by 60% at 100 mg/l (NOEC = 10 mg/l). Developmental retardation occurred at 10 and 100 mg/l (NOEC = 1 mg/l), with lower growth (35% lower body weight at the LOEC), lack of scales and poor pigmentation.	Crane *et al*. 2005
American brook lamprey larvae (*Lampetra appendix*) were treated with perchlorate at 100 or 500 mg/l for 117 d. Both concentrations induced metamorphosis (NOEC < 100 mg/l), and serum concentrations of T4 and T3 declined by 64-76 and 93-96%, respectively.	Holmes *et al*. 1999
Larval sea lampreys (*Petromyzon marinus*) were treated with 500 mg/l perchlorate, and either T4 or T3. Perchlorate alone induced metamorphosis after 8 weeks, but perchlorate treatment in combination with thyroid hormone failed to induce metamorphosis.	Kao *et al*. 1999
Juvenile male zebrafish (*Danio rerio*) were exposed to measured concentrations of perchlorate at 10 and 100 mg/l for up to 90 d. Thyroid colloid area reduced by nearly 50% at 100 mg perchlorate/l after 90 d, and colloid area expressed as a % of follicle area decreased after exposure to 10 mg perchlorate/l for 30 d. Epithelial cell height increased by about 10% after 10 d exposure to 10 mg/l. Finally, thyroid follicle angiogenesis (development of new blood vessels) had increased after 60 d exposure to 10 mg/l. Overall, the 10-90 d NOEC for thyroid histopathology was < 10 mg/l.	Liu *et al*. 2006
Zebrafish larvae (*Danio rerio*) were exposed from 6 to 37 days post-fertilisation to a measured concentration of perchlorate, 120.6 mg/l. This caused a 60% increase in thyroid epithelial cell height (*i.e.* hypothyroidism) and a decrease in thyroid colloid area. This was accompanied by a 40% reduction in fry growth after 46 d. Cessation of perchlorate treatment led to recovery of thyroid histopathology, but not normal colloid area or growth rate.	Liu *et al*. 2008
Sea lampreys (*Petromyzon marinus*) were exposed to perchlorate as the sodium or potassium salt. This caused significant lowering of serum thyroid hormone, and metamorphosis was induced in some larvae.	Manzon *et al*. 2001 [paper not seen]
Larval sea lampreys (*Petromyzon marinus*) were exposed to perchlorate at 100 mg/l for up to 24 weeks. Precocious metamorphosis was observed in treated fish after 8-24 weeks, and T4 and T3 were 62% and 72% reduced, respectively.	Manzon & Youson 1997 [paper not seen]
Larval sea lampreys (*Petromyzon marinus*) were exposed to perchlorate for 23 weeks. Serum T4 was elevated in larvae held at 0.74 µg/l in cold water (3°C) but not warm water (18°C), and metamorphosis was induced in all larvae at the higher	Manzon & Youson 1999 [paper not

temperature but not the lower.	seen]
Adult and sub-adult zebrafish (*Danio rerio*) were exposed to perchlorate at measured concentrations of 0, 11, 90, 1131 and 11,480 µg/l for 12 weeks, and were then allowed to recover in clean water for another 12 weeks. At 12 weeks of exposure, NOECs for thyroid colloid depletion, hypertrophy, angiogenesis and reduction in colloid T4 were 1131, 90, 11 and <11 µg/l, respectively. Angiogenesis and colloid T4 reduction in fish at the top concentration had not returned to normal after 12 weeks recovery. Body growth was not affected by any PER concentration.	Mukhi *et al*. 2005
Mixed-sex populations of adult zebrafish (*Danio rerio*) were exposed to nominal PER concentrations of 0, 10 and 100 mg/l for up to 16 weeks. At 10 weeks, both concentrations had caused thyroidal hypertrophy and colloid depletion. When the fish started reproducing, both perchlorate treatments caused a big reduction (~70% at the LOEC) in packed egg volume (NOEC < 10 mg/l), and small increases in both egg diameter and larval length. Whole-body thyroxine (but not triiodothyronine) titres in mothers and embryos were reduced at both concentrations. By 16 weeks at both concentrations, female body weight had reduced by ~50% (NOEC < 10 mg/l).	Mukhi & Patiño 2007
Adults and fry of mosquitofish (*Gambusia holbrooki*) were exposed to measured concentrations of perchlorate at 1, 10 and 100 mg/l for 8 and 4 weeks, respectively. Growth (weight) of fry was increased by ~60% at 1 mg/l but depressed at 10 mg/l and unchanged at 100 mg/l. Fecundity and GSI of adults were increased (by ~20% at the LOEC) at all concentrations (NOEC < 1 mg/l). The 5 d LC50 for fry was 404 mg/l, so it is unlikely that the reported effects were caused by non-specific toxicity.	Park *et al*. 2006
Zebrafish embryos (*Danio rerio*) were exposed to perchlorate from 2 to 5 d post-fertilisation. This abolished immuno-reactivity to T4 in the thyroid gland follicles, as did T3 treatment.	Raldua & Babin 2009 [paper not seen]
A cyprinid fish, the central stoneroller (*Campostoma anomalum*), was sampled from streams contaminated with perchlorate. Fish from contaminated sites had increased thyroid follicular hyperplasia, hypertrophy, and colloid depletion.	Theodorakis *et al*. 2006
<u>Studies in wildlife species : Amphibians</u>	
Spadefoot toad larvae (*Spea multiplicata*) exposed to measured perchlorate concentrations in natural water up to 1038 µg/l over 42 d experienced no effects on metamorphosis. A similar lack of effects was observed in clawed frogs (*Xenopus laevis*) exposed to measured perchlorate concentrations in natural water of 27 and 57 µg/l. However, *X. laevis* (stage 11 – 66) exposed to perchlorate in FETAX medium showed reduction in % metamorphosis (NOEC = 27 µg/l), increased time to metamorphosis (NOEC = 27 µg/l) and reduction in hind-limb length (NOEC = 27 µg/l). These results were interpreted to mean that something in the natural water was able to mitigate the anti-metamorphic effects of perchlorate.	Brausch *et al*. 2010
Eggs and larvae of the clawed frog (*Xenopus laevis*) were exposed to perchlorate for 70 d. This significantly inhibited tail resorption, fore-limb emergence and hind-limb growth. Whole-body thyroxine content was reduced whereas thyroid follicle	Carr & Goleman 2001 [paper not

cell height was increased.	seen]
This review concludes that no firm links have been established between perchlorate (and other chemical)–induced alterations in the hypothalamus-pituitary-thyroid (HPT) axis and impacts on amphibian or fish populations, but that perchlorate is the only chemical for which a direct link has been established between HPT disruption and adverse effects on fish and amphibian development or reproduction in the laboratory.	Carr & Patiño 2011
Bullfrog larvae (*Rana catesbeiana*) collected from a perchlorate -contaminated site showed a 5-fold reduction in hindlimb and snout-vent length by comparison with larvae from a reference site. Furthermore, the volume of the thyroid gland was reduced.	Carr *et al*. 2003 [paper not seen]
Embryos and larvae of the clawed frog (*Xenopus laevis*) were exposed to measured perchlorate concentrations of 59 and 14,140 µg/l for 70 d followed by 28 d non-treatment recovery. After the recovery period, both concentrations had depressed tail resorption (NOEC < 59 µg/l), while forelimb emergence was delayed at the top concentration (NOEC = 59 µg/l), both before (no emergence) and after recovery (40% reduction). Both concentrations caused hypertrophy of the thyroid follicular epithelium, and skewed the sex ratio towards a lower % of males at metamorphosis (from 42% male to 33 and 26% for 59 and 14,140 µg/l, respectively (NOEC < 59 µg/l).	Goleman *et al*. 2002a
Southern leopard frog larvae (*Rana sphenocephala*) were exposed to nominal concentrations of perchlorate at 15 and 30 mg/l for 15 weeks. The lower concentration caused no mortalities in larvae, but inhibited metamorphosis almost completely (NOEC < 15 mg/l), resulting in high mortality at metamorphosis. The higher concentration was directly lethal.	Ortiz-Santaliestra & Sparling 2007
Early larval gray treefrogs (*Hyla versicolor*) were exposed to perchlorate at 2.2 to 50 mg/l over 70 d. Some inhibition of development was observed at 2.2 mg/l (NOEC < 2.2 mg/l), with almost complete cessation of development (reduced hindlimb formation and metamorphosis) at 22.9 mg/l and above. The 70 d EC50 for total inhibition of metamorphosis was 3.63 mg/l. Co-treatment with small concentrations of iodide was able to counteract the effects of perchlorate.	Sparling *et al*. 2003 [paper not seen]
Cricket frogs (*Acris crepitans*) from perchlorate-contaminated streams did not show evidence of thyroid colloid depletion or hyperplasia, although frogs from the two most contaminated sites showed elevated thyroid follicle cell hypertrophy.	Theodorakis *et al*. 2006
Clawed frog larvae (*Xenopus laevis*) were exposed for 8 d to measured perchlorate at 4 mg/l. Thyroidal 3,5-diodo-L-tyrosine (DIT), 3-mono-diodo-L-tyrosine (MIT) and thyroxine (T4) were all reduced in the perchlorate treatment from Day 2 onwards, followed on Days 6-8 by reductions in plasma T4 and increases in thyroid gland cell hyperplasia.	Tietge *et al*. 2010
Studies in wildlife species: Birds	

Japanese quail chicks (*Coturnix japonica*) were exposed to perchlorate at 2000 mg/l in drinking water for 7.5 weeks beginning on day 5 posthatch. After 2 weeks, hypothyroidism was observed in the form of lower thyroid hormone levels. At later times, there was significant thyroid gland hypertrophy, and the expression of thyroid-responsive genes was affected in liver but not brain.	Chen *et al*. 2009
Northern bobwhite quail chicks (*Colinus virginianus*) were exposed for 8 weeks to perchlorate in drinking water at 0.05, 0.5, 50, 250, 500, 1000, 2000 and 4000 mg/l. Thyroidal T4 was reduced early on at 0.5 mg/l and above (NOEC = 0.05 mg/l), but the NOEC increased to 50 mg/l after 8 weeks, which was taken as evidence of adaptation to perchlorate. Thyroid weight only increased at 500-1000 mg/l (NOEC = 250-500 mg/l).	McNabb *et al*. 2004a
Northern bobwhite quail chicks (*Colinus virginianus*) were exposed to perchlorate at 50-4000 mg/l in drinking water for up to 8 weeks. Decreased thyroidal T4 was the most sensitive measure of effect (30% decrease at the LOEC), with a 2 week NOEC of 0.05 mg/l. The NOEC for increased thyroid weight was 250 mg/l, while the 8 week NOEC for decreased limb growth was 2000 mg/l.	McNabb *et al*. 2004b
Zebra finch chicks (*Taeniopygia guttata*) were orally dosed on posthatch days 3-14 with perchlorate at 10, 100 or 1000 µg/g body weight and effects examined over 72 d. Body weight and leg length were reduced at 100 and 1000 µg/g (NOEC = 10 µg/g), and liver weight increased at the same doses. There were no changes in brain. There were extensive changes in behaviour in the top dose group (NOEC = 100 µg/g), and some behavioural changes (*e.g.* decreased flight attempts) at 10 and 100 µg/g (NOEC < 10 µg/g).	Rainwater *et al*. 2008
Studies in mammalian species	
The ability of potassium perchlorate to promote the carcinogenic activity of N-bis(2-hydroxypropyl)nitrosamine (DHPN) was determined. Rats received no treatment, i.p. injection with DHPN (28 mg/kg body weight) followed by 1000 ppm perchlorate in the diet or 1000 ppm perchlorate in the diet *et al*one (n=20). After 18 weeks, rats treated with DHPN plus perchlorate had 100% incidence of thyroid adenomas whilst controls or rats treated with perchlorate alone had 0% incidence.	Hiasa *et al* (1987).
Ammonium perchlorate was administered to Sprague-Dawley rats (n=6) in drinking water for 14 days at concentrations of 0, 1.25, 5.0, 12.5, 25, 50, 125 or 250 mg/L (corresponding to doses of approximately 0, 0.11, 0.44, 1.11, 2.26, 4.32, 11.44 and 22.16 mg/kg/day). Perchlorate exposure decreased circulating T3 and T4 and increased TSH. Thyroid gland follicular lumen size was decreased and relative thyroid weight increased. At the lowest dose, 0.1 mg/kg/day, statistically significant changes in serum T4 were observed in both sexes.	Caldwell *et al* (1995, reported in NAS, 2005 and CEPA, 2004).
The effects of perchlorate on motor activity in Sprague-Dawley rats were determined. The females were dosed with ammonium perchlorate in drinking water for two weeks at 0, 0.1, 1, 3, or 10 mg/kg/day prior to mating with the untreated males and until PND10. On PND14, one male and one female were randomly selected from each litter to be used in motor activity testing. The same animals	Bekkedal *et al* (2000 reported in NAS, 2005 and CEPA, 2004).

were tested on PND14, PND18, and PND22. The authors reported that there were no reliable effects of treatment on motor activity although there was a divergence in activity between the control and treated groups. A further analysis by US EPA concluded that a dose-related increase in motor activity occurred with a NOAEL of 1 mg/kg/day.	
Inhibition of thyroidal iodine uptake was determined in rats. Sprague-Dawley rats (n=6) were injected with perchlorate at 0, 0.01, 0.1, 1 or 3 mg/kg to groups of male rats At 2 hr post dosing, the rats were challenged with ^{125}I (33 µg/kg) by intravenous injection and killed at various time points. Inhibition of thyroidal iodide uptake occurred in 1 and 3 mg/kg dose groups after 2, 6, and 9 hr. Inhibition was also observed at 0.1 mg/kg dose after 9 hr. Similar results were obtained during a study where perchlorate was administered in drinking water at 0, 1, 3, and 10 mg/kg/day for up to 14 days.	Yu *et al* (2000, reported in NAS, 2005 and CEPA, 2004).
Male rats (n=8) were administered perchlorate in drinking water at 0, 0.1, 1, 3, and 10 mg/kg/day for 1,5 or 14 days. In all treated groups TSH levels were increased compared to controls. Serum T4 levels were initially decreased in all dose groups except 0.1 mg/kg/day. By 14 days, the 1 mg/kg/day dose group returned to control T4 values while T4 levels of the 3 and 10 mg/kg-day dose groups were still significantly depressed.	Yu *et al* (2000, reported in NAS, 2005 and CEPA, 2004).
Ammonium perchlorate was tested in a developmental neurotoxicity study. Pregnant Sprague-Dawley rats (n=25) were administered doses of 0, 0.1, 1.0, 3.0, and 10 mg/kg/day via drinking water from GD 0 to PND 22. Perchlorate had no effect on body weights, feed consumption, clinical observations, or sexual maturation of pups at all exposures levels. Analysis of the F1 data concluded that perchlorate treatment was associated with: (a) brain morphometric changes in the 10 mg/kg-day dose group and possibly also the 3 mg/kg/day dose group; (b) thyroid colloid depletion, hypertrophy and hyperplasia in the 0.1 and 3 mg/kg/day dose groups; (c) thyroid hormone (T3 and T4) changes in the 0.1 and 1 mg/kg/day dose groups; and (d) increases in motor activity in some dosed animals.	Argus (1998a, reported in NAS, 2005 and CEPA, 2004); York *et al* (2004, 2005).
Ammonium perchlorate was tested in a rabbit developmental toxicity study. Pregnant New Zealand White rabbits (n=25) were administered doses of 0, 0.1, 1.0, 10, 30 and 100 mg/kg/day via drinking water from GD 6 to GD 28. Fetuses were delivered by Caesarean section. Litter parameters (corpora lutea, implantations, litter sizes, live and dead fetuses, early and late resorptions, % dead or resorbed conceptuses, % male fetuses and fetal body weights) were unaffected at all dose levels. Decreases in maternal T4 occurred at 1, 10, 30, and 100 mg/kg/day but there were no significant changes of T3 or TSH at any dose. No fetal alterations (defined as malformations and variations) were attributable to exposure to ammonium perchlorate at all doses.	Argus (1998b, reported in NAS, 2005 and CEPA, 2004); York *et al* (2001).
Female rats were administered doses of 0, 0.01, 0.1, 1.0 and 30 mg/kg/day via drinking water, beginning 15 days before cohabitation with males and continuing until termination on GD 21. A gross necropsy of the thoracic, abdominal, and pelvic viscera was performed. Preimplantation loss was noted at all dose levels: 12, 18, 20, 16, and 25 % at the respective doses from 0 to 30 mg/kg/day. A decrease in the number of live fetuses was reported at 30 mg/kg/day, although no significant decrease was noted at lower doses. Ossification sites per litter for sternal centers	Argus (2000, reported in NAS, 2005 and CEPA, 2004).

and forelimb phalanges were significantly reduced at 30 mg/kg/day.	
The effects of exposure to a low or high concentration of ammonium perchlorate on follicle maturation in the Long-Evans hooded rat and possible amelioration by T4 supplementation were determined. Animals were treated with perchlorate (0.4 or 4.0 mg/kg/day) via drinking water on GD 7-21. Half of each group was also given T4 supplements via drinking water on GD 7-21. Ovaries were removed from female pups on PND 24/25. Perchlorate at 4.0 mg/kg/day significantly reduced the number of preantral follicles <50,000 μm^2 and the total number of antral follicles in the <50,000, 50-100,000 and >100,000 μm^2 size classes. In ovaries treated with the 0.4 mg/kg/day, there were no decreases in preantral follicles of any size class and only a significant reduction in the largest antral follicles. T4 attenuated the effect on the number of small preantral and antral follicles; however, a significant diminution in the antral follicle number persisted in the mid-sized and large-sized classes.	Baldridge *et al* (2004)
Pregnant Sprague-Dawley rats were administered perchlorate in drinking water (0 or 1 mg/kg/day) from GD 2 to 20. One set of control and exposed dams was killed on GD day 20. The litters from the second set of control and exposed dams were cross-fostered immediately after parturition and were killed on PND day 10. Serum perchlorate levels for control pups cross-fostered to treated dams had serum levels of approx 0.54 µg/ml whilst treated pups cross-fostered to control dams had serum perchlorate levels of approx 0.38 µg/ml. Female pups receiving perchlorate lactationally had significantly lower levels of serum T4 than control pups and prenatally exposed pups. Serum T4 levels in male pups were not affected by perchlorate. Serum thyroid hormone levels from gestational perchlorate exposure were restored to control values by postnatal day 10.	Mahle *et al* (2003)

Toxicokinetics in mammalian species

74. Results of individual studies are not shown. The following information is taken from CEPA (2004):

"Perchlorate appears to be readily absorbed following oral administration and over 90% of the dose is excreted in urine over a 24 h period. It does not appear to undergo metabolism.

6.2 Analysis of Building Blocks Using Perchlorate Data

75. Each assay for which guidance is given in the draft GD has been evaluated using the relevant data for perchlorate inserted into the building block in the draft GD. The approach used is described in Section 3.2 (above). The wording in each series of tables is designed to mirror that used in the building blocks (BBs) in the draft GD. The results of the assays have been abbreviated here for clarity but more detail is given in Tables 5.1 and 5.2.

6.3 Perchlorate Case Study Results: Validated *in vitro* Assays

6.3.1 ER Binding Assay (US EPA OPPTS 890.1250) (GD No. 150 Section C.2.1)

There areno data available

6.3.2 AR Binding Assay (US EPA OPPTS 890.1150) (GD No. 150 Section C.2.2)

There are no data available

6.3.3 OECD TG 455: The Stably Transfected Human ERα Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA) (including Guidance for the Antagonism Assay) (GD No. 150 Section C.2.3)

There are no data available

6.3.4 OECD TG 456: H295R Steroidogenesis Assay (GD No. 150 Section C.2.4)

There are no data available

6.3.4 Aromatase Assay (US EPA OPPTS 890.1200) (GD No. 150 Section C.2.5)

There are no data available

6.4 Perchlorate Case Study Results: Validated Wildlife *in vivo* Assays

6.4.1 OECD TG 229: Fish Short Term Reproduction Assay (FSTRA) (GD No. 150 Section C.3.1)

Scenario J : Result -, existing *in vitro* data +, existing *in vivo* data +

Scenario J is appropriate because the standard assay shows no reproduction-related responses, while existing *in vitro* data show a variety of responses consistent with thyroid interactions (specifically,

inhibited iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with thyroid interference.

Table 6.4.1.1: Perchlorate data summary

Fish Short Term Reproduction Assay data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
A standard assay with fathead minnow (USEPA 2006) showed no effects on VTG or fecundity at up to 44 mg/l although ovarian atresia increased (LOEC = 5.5 mg/l). A non-standard assay (Patiño *et al*. 2003) did show reduced fecundity at a high concentration (NOEC = 18 mg/l) as well as thyroidal abnormalities. As the primary endpoints in the standard assay showed no response, these data have been interpreted as **negative** for the purposes of this exercise.	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011). Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	There are no fish data from TG 230. However, non-standard short-term studies in a variety of fish species reveal a range of thyroid-related effects including thyroid hypertrophy and hyperplasia (Bradford *et al*. 2005; Crane *et al*. 2005; Liu *et al*. 2008), altered thyroid hormone titres (Bradford *et al*. 2005; Crane *et al*. 2005; Raldua and Babin 2009), and developmental retardation / reduced growth (Crane *et al*. 2005; Liu *et al*. 2008). TG 231 (OECD 2007a) gave NOECs of 62.5-125 µg/l for altered growth and thyroid histology in *X. laevis* larvae, while similar assays with the same species (Goleman *et al*. 2002b; Opitz *et al*. 2009; Tietge *et al*. 2005) gave reduced growth and tail resorption, thyroid histopathology and retarded metamorphosis, with NOECs down to 5 µg/l

Fish Short Term Reproduction Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			and below. A non-standard short-term *X. laevis* assay (Tietge *et al*. 2010) gave a NOEC of 4 mg/l for effects on thyroid hormone levels and thyroid histopathology.
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic *Xenopus laevis* tadpoles (Hornung *et al* 2010).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991).	
		TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion)	

Fish Short Term Reproduction Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000).	
		An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000).	
		No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day)	

Fish Short Term Reproduction Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004.	

Table 6.4.1.2: Conclusions about Fish Short-Term Reproduction Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase

evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for perchlorate	Next Step (as BB)	Assay result for perchlorate
Based on the existing data, the chemical has endocrine activity *in vivo*. The lack of response in OECD TG 229 suggests that fish are not responsive, unless the existing data are from fish	If existing *in vivo* data are from fish, consider performing a Fish Sexual Development Test (unless reproduction is known to be the most sensitive life-stage).	No standard FSDT data are available. However, an assay similar to TG 234 with zebrafish (Mukhi *et al*. 2007) suppressed growth, skewed the sex ratio towards females, and induced hypothyroidism (unbounded LOEC = 100 mg/l for all effects). These results suggest that perchlorate may be an ED in fish, but only at very high concentrations. Furthermore, a range of other fish partial lifecycle data from non-standard assays support this conclusion (Holmes *et al*. 1999; Kao *et al*. 1999; Liu *et al*. 2006; Manzon and Youson, 1999; Mukhi *et al*. 2005; Mukhi & Patiño 2007).

Table 6.4.1.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other considerations (as given in BB)	Conclusions for Perchlorate
None	n/a

76. Overall conclusions about Fish Short-Term Reproduction Assay and existing data: Although the standard TG 229 assay data did not give a clear response which could be firmly attributed to endocrine disruption, the conclusion (on the basis of existing *in vitro* data with fish/amphibian/mammalian cell-lines and tissues, and existing *in vivo* data in mammals, fish and amphibians) to proceed to an FSDT was vindicated, as this and related partial lifecycle studies show that perchlorate is able to skew fish sex ratios as well as cause a range of interferences with the thyroid system. This reveals the importance of considering the weight of all available and relevant evidence. However, it seems clear from these data that perchlorate is probably only a weak ED in fish, with NOECs in the mg/l range.

6.4.2 OECD TG 230: 21 Day Fish Assay (GD No. 150 Section C.3.2)

No data are available

6.4.3 Variant of OECD TG 230: Androgenised Female Stickleback Screen (AFSS) (GD No. 150 Section C.3.3)

No data are available.

6.4.4 Fish sexual development test (FSDT) (TG 234) (GD No. 150 Section C.3.4)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows suppressed growth, ♀-skewed sex ratio and hypothyroidism, while existing *in vitro* data show a variety of responses consistent with thyroid interactions (specifically, inhibited iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with thyroid interference.

Table 6.4.4.1: Perchlorate data summary

Fish Sexual Development Test data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
No standard FSDT data are available. However, an assay similar to TG 234 with zebrafish (Mukhi *et al*. 2007) suppressed growth, skewed the sex ratio towards females, and induced hypothyroidism (unbounded LOEC = 100 mg/l for all effects).	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	A standard TG 229 assay with fathead minnow (USEPA 2006) showed no effects on VTG or fecundity at up to 44 mg/l although ovarian atresia increased (LOEC = 5.5 mg/l). However, a non-standard assay (Patiño *et al*. 2003) did show reduced fecundity at a high concentration (NOEC = 18 mg/l) as well as thyroidal abnormalities.
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).		
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic Xenopus laevis tadpoles (Hornung *et al* 2010).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking	A range of fish partial lifecycle data from non-standard assays support the conclusion that perchlorate can interfere with the thyroid system in fish at high concentrations (Holmes *et al*. 1999; Kao *et al*. 1999; Liu *et al*. 2006; Manzon and Youson, 1999; Mukhi *et al*. 2005; Mukhi & Patiño 2007). Data from TG 231

Fish Sexual Development Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991).	(OECD 2007a) and related assays (Goleman *et al*. 2002b; Opitz *et al*. 2009; Tietge *et al*. 2005) show that PER produces short-term thyroid system effects in amphibian larvae. Longer-term non-standard assays with amphibians (Brausch *et al*. 2010; Carr and Goleman, 2001; Goleman *et al*. 2002a; Olmstead, 2009; Ortiz-Santaliestra & Sparling 2007; Sparling *et al*. 2003) also show a range of effects attributable to thyroid disruption, with NOECs as low as 0.027 mg/l. Finally, non-standard partial lifecycle studies with birds (Chen *et al*. 2009; McNabb *et al*. 2004a&b; Rainwater *et al*. 2008) observed a range of thyroid-related effects including hypothyroidism, thyroid hypertrophy, reduced limb growth, and abnormal behaviour. NOECs for these effects ranged down to 10 µg/g body weight (oral dosing) and below, or 0.05 mg/l in drinking water.
		TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	

Fish Sexual Development Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001).	

Fish Sexual Development Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004.	
		Male PP assay- in rats dosed by gavage, T4 was decreased, TSH was increased, whilst T3 was unaffected at doses from 125 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased from 62.5 mg/kg/day. No other endocrine-related endpoints were affected (Stoker *et al* 2006).	

Fish Sexual Development Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	

Table 6.4.4.2: Conclusions about Fish Sexual Development Test and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for Perchlorate	Next Step (as BB)	Assay result for Perchlorate
Strong evidence for adverse effects in fish and other organisms by an endocrine mechanism.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish lifecycle test should be considered.	A standard lifecycle test with medaka (ABC 2009) run at perchlorate concentrations up to 1 mg/l showed no effects on growth or reproduction at any concentration. However, some female fish showed thyroid epithelial hypertrophy and colloid depletion (overall NOEC = 0.27 mg/l). A non-standard test with 3-spined stickleback (Bernhardt *et al*. 2006) has been run for almost a whole lifecycle (F0 adulthood to F1 reproductive maturity) at higher concentrations than the study with medaka. This showed that male nuptial colouration, courtship behaviour and normal sexual development were all depressed (unbounded NOEC = 30 mg/l). Furthermore, some females were masculinised, with ovotestes, and

Conclusions for Perchlorate	Next Step (as BB)	Assay result for Perchlorate
		the sperm from these fish was infertile. This confirms that longer-term assays than the FSDT, but at high concentrations, also show adverse effects related to endocrine disruption.

Table 6.4.4.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other considerations (as given in BB)	Conclusions for perchlorate
If the FSDT was only performed with 3 test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case the FSDT would be less responsive than a lifecycle test.	The available FSDT was only performed with 2 test concentrations, so is certainly not appropriate for establishing a reliable NOEC. The available lifecycle test data are insufficient for judging whether reproduction is more sensitive than sexual development.
A decision about whether to conduct FLCTT or MMGT may be driven primarily by the bioaccumulative properties of the chemical – a one generation test (*e.g.* F0 eggs to F1 fry) will generally be sufficient if the chemical is not expected to be transferred to the fry via the eggs.	No MMGT data are available. However, perchlorate would not be expected to bioaccumulate strongly, so may not be more potent in an MMGT than an FLCTT.

77. Overall conclusions about Fish Sexual Development Test and existing data: Both the FSDT assay data and the other available *in vitro* and *in vivo* information predict that perchlorate is able to cause endocrine disturbances in fish which can have various adverse impacts as high (mg/l) concentrations, and this prediction is supported by results from fish (stickleback) semi-lifecycle testing, thus underpinning the recommendation to conduct such testing if more evidence is needed. However, note that sub-mg/l concentrations only cause thyroid histopathology and no apical effects in fish (medaka). Given the availability of the amphibian data which indicate that this group is more sensitive than fish (short-term NOECs in the µg/l range), it might be more appropriate to conduct a LAGDA, or a full amphibian lifecycle study (if a suitable lifecycle TG were available).

6.4.5 Fish Lifecycle Toxicity Test (FLCTT) (USEPA OPPTS 850.1500) (GD No. 150 Section C.3.5)

Scenario J: Result -, existing *in vitro* data +, existing *in vivo* data +

Scenario J is appropriate because the assay shows no significant effects on growth or reproduction, while existing *in vitro* data show a variety of responses consistent with thyroid interactions (specifically, inhibited iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with thyroid interference.

Table 6.4.5.1: Perchlorate data summary

Fish Lifecycle Toxicity Test data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
A standard assay with medaka (ABC 2009) showed no significant apical effects on either growth or reproduction at any concentration (NOEC >1 mg/l). However, there were some effects on female thyroid histopathology (overall NOEC = 0.27 mg/l).	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011). Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002). Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic *Xenopus laevis* tadpoles (Hornung *et al* 2010).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000). TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and	A standard TG 229 assay with fathead minnow (USEPA 2006) showed no effects on VTG or fecundity at up to 44 mg/l although ovarian atresia increased (LOEC = 5.5 mg/l). However, a non-standard assay (Patiño *et al*. 2003) did show reduced fecundity at a high concentration (NOEC = 18 mg/l) as well as thyroidal abnormalities. A range of fish partial lifecycle data from non-standard assays support the conclusion that perchlorate can interfere with the thyroid system in fish at high concentrations (Holmes *et al*. 1999; Kao *et al*. 1999; Liu *et al*. 2006; Manzon and Youson, 1999; Mukhi *et al*. 2005; Mukhi & Patiño 2007). A non-standard semi-lifecycle test with 3-spined stickleback (Bernhardt *et al*. 2006) has been run for almost

Fish Lifecycle Toxicity Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991). TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001). Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987). In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995).	a whole lifecycle (F0 adulthood to F1 reproductive maturity) at higher concentrations than the study with medaka. This showed that male nuptial colouration, courtship behaviour and normal sexual development were all depressed (unbounded NOEC = 30 mg/l). Furthermore, some females were masculinised, with ovotestes, and the sperm from these fish was infertile. Data from TG 231 (OECD 2007a) and related assays (Goleman *et al*. 2002b; Opitz *et al*. 2009; Tietge *et al*. 2005) show that PER produces short-term thyroid system effects in amphibian larvae. Longer-term non-standard assays with amphibians (Brausch *et al*. 2010; Carr and Goleman, 2001; Goleman *et al*. 2002a; Olmstead, 2009; Ortiz-Santaliestra & Sparling 2007; Sparling *et al*. 2003) also show a range of effects attributable to thyroid disruption, with NOECs as low as 0.027 mg/l. Finally, non-standard partial lifecycle studies

Fish Lifecycle Toxicity Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during	with birds (Chen *et al*. 2009; McNabb *et al*. 2004a&b; Rainwater *et al*. 2008) observed a range of thyroid-related effects including hypothyroidism, thyroid hypertrophy, reduced limb growth, and abnormal behaviour. NOECs for these effects ranged down to 10 µg/g body weight (oral dosing) and below, or 0.05 mg/l in drinking water.

Fish Lifecycle Toxicity Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004). Male PP assay- in rats dosed by gavage, T4 was decreased, TSH was increased, whilst T3 was unaffected at doses from 125 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased from 62.5 mg/kg/day. No other endocrine-related endpoints were affected (Stoker *et al* 2006). Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	

Table 6.4.5.2: Conclusions about Fish Life Cycle Toxicity Test and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for perchlorate	**Next Step (as BB)**	**Assay result for perchlorate**
The chemical is probably not an ED in fish, unless this conclusion is contradicted by existing *in vivo* data.	If the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, consider conducting an MMGT.	No MMGT data are available. However, it should be noted that perchlorate is not bioaccumulative in fish and is not suspected of epigenetic effects, so it is unlikely that an MMGT will be more sensitive than an FLCTT.

Table 6.4.5.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other considerations (as given in BB)	**Conclusions for perchlorate**
If any effects in an MMGT can be plausibly linked with mechanistic data, the test chemical is probably an ED.	No MMGT data are available.

78. Overall conclusions about Fish Life Cycle Toxicity Test and existing data: The conclusions in the guidance document are supported by the available evidence (*i.e.* a conclusion from this FLCTT that perchlorate is not an ED is contradicted by evidence from other assays). Although this assay showed no adverse apical effects at the concentrations tested, other partial- and semi-lifecycle fish data revealed a range of such effects at higher concentrations, and a biomarker (thyroid histopathology) in the present assay also indicated interference with thyroid function. These results highlight the fact that data from a single assay may be misleading, that test concentrations must be carefully chosen, and that it is important to consider the overall weight of evidence.

6.4.6 OECD TG 231: Amphibian Metamorphosis Assay (AMA) (GD No. 150 Section C.3.6)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows decreased limb length and thyroidal hypertrophy, while existing *in vitro* data show a variety of responses consistent with thyroid interactions (specifically, inhibited iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with thyroid interference.

Table 6.4.6.1: Perchlorate data summary

Amphibian Metamorphosis Assay data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
A standard TG 231 assay (OECD 2007a) showed increases in growth (NOEC = 62.5-125 µg/l), decreases in hind limb length (NOEC = 125 µg/l), and thyroidal hypertrophy (NOEC <62.5-62.5 µg/l). Similar, but non-standard, assays with *X. laevis* (Goleman *et al*. 2002b; Opitz *et al*. 2009; Tietge *et al*. 2005) generally supported these observations, with NOECs ranging from < 5 µg/l to 63 µg/l.	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	A standard TG 229 assay with fathead minnow (USEPA 2006) showed no effects on VTG or fecundity at up to 44 mg/l although ovarian atresia increased (LOEC = 5.5 mg/l). A non-standard assay (Patiño *et al*. 2003) did show reduced fecundity at a high concentration (NOEC = 18 mg/l) as well as thyroidal abnormalities. Non-standard short-term studies in a variety of fish species reveal a range of thyroid-related effects including thyroid hypertrophy and hyperplasia (Bradford *et al*. 2005; Crane *et al*. 2005; Liu *et al*. 2008), altered thyroid hormone titres (Bradford *et al*. 2005; Crane *et al*. 2005; Raldua and Babin

Amphibian Metamorphosis Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			2009), and developmental retardation / reduced growth (Crane *et al*. 2005; Liu *et al*. 2008). Finally, a non-standard short-term *X. laevis* assay (Tietge *et al*. 2010) gave a NOEC of 4 mg/l for effects on thyroid hormone levels and thyroid histopathology.
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991).	
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic Xenopus laevis tadpoles (Hornung *et al* 2010).	TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day	

Amphibian Metamorphosis Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		(endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000).	
		An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000).	

Amphibian Metamorphosis Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004).	

Table 6.4.6.2: Conclusions about Amphibian Metamorphosis Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for perchlorate	**Next Step (as BB)**	**Assay result for perchlorate**
Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians, plus thyroid effects in other species	Consider performing a Larval Amphibian Growth and Development Assay (LAGDA).	A LAGDA-type test with *Xenopus tropicalis* (Olmstead, 2009) produced thyroid histopathology (NOEC = 170 µg/l) and mild VTG induction in females, but no apical endpoints (*i.e.* metamorphosis, body size or gonadosomatic indices) were affected at concentrations up to 1500 µg/l. This suggest either that the short-term effects on growth seen in *X. laevis* are not serious enough to be translated into apical effects in adults, or that the two species have differing sensitivities.

Table 6.4.6.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other considerations (as given in BB)	**Conclusions for perchlorate**
Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.	The data indicate that perchlorate inhibits iodide uptake and therefore acts as a *de facto* thyroid antagonist, although it does not operate via the thyroid receptor.

79. Overall conclusions about Amphibian Metamorphosis Assay and existing data:

The evidence from the LAGDA-type test with *X. tropicalis* suggests that the results of TG 231 (which showed apical short-term effects on the growth of *X. laevis* larvae) are not translated into adverse apical effects in adults at the concentrations investigated. This result may be caused by interspecies differences in sensitivity, but it also seems likely that larvae are able to cope with a certain degree of thyroid disruption without long-term adverse consequences. It is also possible that long-term exposure to higher concentrations of perchlorate than were tested in the LAGDA-type test would cause adverse effects,

especially if reproductive success were to be an endpoint. Certainly, a number of non-standard long-term tests with other amphibian species at concentrations in the mg/l range do show a range of adverse effects including reduced metamorphosis and growth. Overall, the advice to conduct a LAGDA was sound, but it shows that long-term effects predicted on the basis of a short-term screen may not necessarily be realised in practice.

6.4.7 OECD TG 206: Avian Reproduction Test (GD No. 150 Section C.3.7)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay shows suppressed growth and reduction in eggshell thickness, while existing *in vitro* data show a variety of responses consistent with thyroid interactions (specifically, inhibited iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with thyroid interference.

Table 6.4.7.1: Perchlorate data summary

Avian Reproduction Test data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
A standard assay with Japanese quail (*Coturnix japonica*) (Springborn Laboratories, 2011) revealed a small reduction in eggshell thickness (to 91% of the control value at the LOEC) with a NOEC of 100 mg/kg feed, and a reduction in female weight gain (to 76% of the control value at the LOEC) with a NOEC of 500 mg/kg feed.	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	A standard TG 229 assay with fathead minnow (USEPA 2006) showed no effects on VTG or fecundity at up to 44 mg/l although ovarian atresia increased (LOEC = 5.5 mg/l). However, a non-standard assay (Patiño *et al*. 2003) did show reduced fecundity at a high concentration (NOEC = 18 mg/l) as well as thyroidal abnormalities. A range of fish partial lifecycle data from non-standard assays support the conclusion that perchlorate can interfere with the thyroid system in fish at high concentrations
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).		
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic Xenopus laevis tadpoles (Hornung *et al* 2010).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was	

Avian Reproduction Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991). TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	(Holmes *et al*. 1999; Kao *et al*. 1999; Liu *et al*. 2006; Manzon and Youson, 1999; Mukhi *et al*. 2005; Mukhi & Patiño 2007). Data from TG 231 (OECD 2007a) and related assays (Goleman *et al*. 2002b; Opitz *et al*. 2009; Tietge *et al*. 2005) show that perchlorate produces short-term thyroid system effects in amphibian larvae. Longer-term non-standard assays with amphibians (Brausch *et al*. 2010; Carr and Goleman, 2001; Goleman *et al*. 2002a; Ortiz-Santaliestra & Sparling 2007; Sparling *et al*. 2003) also show a range of effects attributable to thyroid disruption, with NOECs as low as 0.027 mg/l. Finally, non-standard partial lifecycle studies with birds (Chen *et al*. 2009; McNabb *et al*. 2004a&b; Rainwater *et al*. 2008) observed a range of thyroid-related effects including hypothyroidism, thyroid hypertrophy, reduced limb growth, and abnormal behaviour. NOECs for these effects ranged

Avian Reproduction Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
			down to 10 µg/g body weight (oral dosing) and below, or 0.05 mg/l in drinking water.
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at	

Avian Reproduction Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking	

Avian Reproduction Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004).	
		Male PP assay- in rats dosed by gavage, T4 was decreased, TSH was increased, whilst T3 was unaffected at doses from 125 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased from 62.5 mg/kg/day. No other endocrine-related endpoints were affected (Stoker *et al* 2006).	

Avian Reproduction Test data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	

Table 6.4.7.2: Conclusions about Avian Reproduction Test and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for Perchlorate	Next Step (as BB)	Assay result for Perchlorate
The test chemical is probably an ED if the modality identified in existing screens/tests can be plausibly linked to the affected endpoint.	Further evidence is probably not required.	n/a

Table 6.4.7.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other considerations (as given in BB)	**Conclusions for perchlorate**
If the affected endpoint in OECD TG 206 cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED in birds.	It is plausible that the reduced weight gain seen at the highest concentration is due to interference with the thyroid system, as revealed in *in vitro* screens and *in vivo* tests with fish and amphibians.
OECD TG 206 cannot detect effects on sexual development and is unlikely to detect epigenetic effects or effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them.	No ATGT data are available. However, long-term effects due to epigenesis or bioaccumulation in eggs are unlikely for perchlorate.

80. Overall conclusions about Avian Reproduction Test and existing data: The GD conclusions that the test substance is probably an ED if the effects can be linked an endocrine modality, and that further evidence is probably not required, are supported by the other available data. These show that perchlorate acts by inhibiting iodide uptake and hence thyroid hormone production, and that birds experience thyroid histopathology when orally dosed with relatively high concentrations of perchlorate, leading to reduced growth in *C. japonica* (but not in *C. virginianus*). Similar effects (also at relatively high perchlorate concentrations) may be observed in fish and mammals, and amphibians also show related effects at lower concentrations. On the other hand, if no other data were available, it is clear that the results of TG 206 could not in themselves lead to a conclusion that perchlorate is an ED.

6.5 Perchlorate Case Study Results: Validated Mammalian *in vivo* Assays

6.5.1 OECD TG 440: Uterotrophic Bioassay in Rodents (UT assay) (Including GD on the Use of the Assay to Screen for Anti-Estrogenicity) (GD No. 150 Section C.4.1)

No data are available

6.5.2 OECD TG 441: Hershberger Bioassay in Rats (H Assay) (Including OECD GD for Weanling Hershberger Bioassay) (GD No. 150 Section C.4.2)

No are data available

6.5.3 Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats (Male PP Assay) (US EPA OPPTS 890.1500) (GD No. 150 Section C.4.3)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay was positive, showing changes in thyroid hormones and alteration of thyroid follicular histology. Existing *in vitro* data show a variety of responses consistent with thyroid interactions (inhibition of iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with disruption of the hypothalamus- pituitary-thyroid axis.

Table 6.5.3.1: Perchlorate data summary

Male Rat Pubertal Development and Thyroid Function Assay data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Perchlorate was positive in the male pubertal rat assay. T4 was decreased in a dose-dependent manner from 125 mg/kg/day, TSH was increased at the same doses, whilst T3 was unaffected. Thyroid histology was significantly altered at all doses: a clear dose-dependent decrease in colloid area and increase in follicular cell height. No effects on preputial separation or reproductive tissues weight were observed. (Stoker *et al*, 2006).	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	No data
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991).	

Male Rat Pubertal Development and Thyroid Function Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic *Xenopus laevis* tadpoles (Hornung *et al* 2010).	TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al*	

Male Rat Pubertal Development and Thyroid Function Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10	

Male Rat Pubertal Development and Thyroid Function Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		(Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004.	
		Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	

Table 6.5.3.2: Conclusions about Male Rat PP Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for Perchlorate	Next Step (as BB)	Assay Result for Perchlorate
Increased evidence that perchlorate has activity as a thyroid disrupter (weak, moderate or strong).	Perform assay from CF level 5 *e.g.* ext-1 or 2-gen assay.	Existing 2-gen study: positive for effects on thyroid (Argus 1999; York *et al*, 2001).

Table 6.5.3.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other Considerations (as given in BB)	Conclusions for Perchlorate
If existing data are from level 5 then there is sufficient information to conclude evidence of	Level 5 2-gen assay on perchlorate provides evidences of concern with respect to the thyroid gland only. No

Other Considerations (as given in BB)	Conclusions for Perchlorate
concern for endocrine disruption (the ext-1 gen assay provides the most information).	developmental and reproductive effects were seen but sensitive endpoints of endocrine disruption *e.g.* sexual maturity, were not measured. There is some evidence from developmental neurotoxicity studies that perchlorate has effects on the brain. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention). A number of endpoints sensitive to disruption of the HPT axis are also included and these provide alerts to possible effects on the developing brain as well as the thyroid. A cohort to include an assessment of the developing nervous system is also part of TG 443.
Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints.	Consideration is valid but perchlorate affects both indicators of hormonal activity and apical endpoints.
Effects on apical endpoints alone may indicate EATS modalities or other mechanisms.	Consideration is valid but perchlorate affects both indicators of hormonal activity and apical endpoints.
Possible effects on E modality should also be considered.	Consideration is valid. There are no data on the effect of perchlorate in *in vitro* ER –based assays, or in the uterotrophic assay.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Perchlorate was given orally in most studies. The available data indicates that it is well absorbed and not metabolized. The existing "*in vivo* effects of concern"do not suggest any ADME issues.

81. Overall conclusions about Male Rat PP Assay and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence of concern for endocrine activity in mammals, with respect to disruption of thyroid hormones. The possibility of further tests to provide a greater understanding of effects on development, especially in the brain, are prompted by the guidance. The effects seen in mammals also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different.

6.5.4 Pubertal Development and Thyroid Function Assay in Peripubertal Female Rats (Female PP Assay) (US EPA OPPTS 890.1450) (GD No. 150 Section C.4.4)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay was positive, showing changes in thyroid hormones and alteration of thyroid follicular histology. Existing *in vitro* data show a variety of responses consistent with thyroid interactions (inhibition of iodide uptake and T4 release), and existing *in*

vivo data show a variety of endpoints consistent with disruption of the hypothalamus- pituitary-thyroid axis.

Table 6.5.4.1: Perchlorate data summary

Female Rat Pubertal Development and Thyroid Function Assay data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Perchlorate was positive in the female pubertal rat assay. T3 and 4 were decreased in a dose-dependent manner from 125 mg/kg/day, TSH was increased at 250 and 500 mg/kg. Thyroid histology was significantly altered at all doses with a dose-dependent decrease in colloid area and increase in follicular cell height. No effects on vaginal opening or weights of uterus and ovaries were observed. There were no treatment-related effects on estrous cyclicity. (US EPA 2007, Laws 2011).	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	No data
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were	

Female Rat Pubertal Development and Thyroid Function Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991).	
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic Xenopus laevis tadpoles (Hornung *et al* 2010).	TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There	

Female Rat Pubertal Development and Thyroid Function Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and	

Female Rat Pubertal Development and Thyroid Function Assay data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004.	
		Male PP assay- in rats dosed by gavage, T4 was decreased, TSH was increased, whilst T3 was unaffected at doses from 125 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased from 62.5 mg/kg/day. No other endocrine-related endpoints were affected (Stoker *et al* 2006).	

Table 6.5.4.2: Conclusions about Female Rat PP Assay and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for Perchlorate	Next Step (as BB)	Assay Result for Perchlorate
Increased evidence that perchlorate has activity as a thyroid disrupter (weak, moderate or strong).	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	Existing 2-gen study: positive for effects on thyroid (Argus 1999; York *et al*, 2001).

Table 6.5.4.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other Considerations (as given in BB)	**Conclusions for Perchlorate**
If existing data are from level 5 then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Level 5 2-gen assay on perchlorate provides evidences of concern with respect to the thyroid gland only. No developmental and reproductive effects were seen but sensitive endpoints of endocrine disruption *e.g.* sexual maturity, were not measured. There is some evidence from developmental neurotoxicity studies that perchlorate has effects on the brain. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention). A number of endpoints sensitive to disruption of the HPT axis are also included and these provide alerts to possible effects on the developing brain as well as the thyroid. A cohort to include an assessment of the developing nervous system is also part of TG 443.
Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints.	Consideration is valid but perchlorate affects both indicators of hormonal activity and apical endpoints.
Effects on apical endpoints alone may indicate EATS modalities or other mechanisms.	Consideration is valid but perchlorate affects both indicators of hormonal activity and apical endpoints.
Possible effects on A modality should also be considered.	Consideration is valid. There are no data on the effect of perchlorate in *in vitro* AR –based assays, or in the Hershberger assay.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Perchlorate was given orally in most studies. The available data indicates that it is well absorbed and not metabolized. The existing "*in vivo* effects of concern" do not suggest any ADME issues.

82. Overall conclusions about Female Rat PP Assay and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence of concern for endocrine activity in mammals, with respect to disruption of thyroid hormones. The possibility of further tests to provide a greater understanding of effects on development, especially in the brain, are prompted by the guidance. The effects seen in mammals also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different.

6.5.5 OECD TG 407: Repeated Dose 28 Day Oral Toxicity Study in Rodents (GD No. 150 Section C.4.5)

No data are available

6.5.6 OECD TG 416: Two-Generation Reproduction Toxicity Study (GD No. 150 Section C.4.6)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay was positive for effects on the thyroid, showing changes in thyroid hormones and alteration of thyroid follicular histology. Existing *in vitro* data show a variety of responses consistent with thyroid interactions (inhibition of iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with disruption of the hypothalamus- pituitary-thyroid axis.

Table 6.5.6.1: Perchlorate data summary

Two-Generation Reproduction Toxicity Study data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Perchlorate was positive for effects on the thyroid in a rat two generation assay. No effects on reproduction or development were noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). There were also no effects on sperm parameters or estrous cyclicity in P or F1 generations. Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion)	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	No data
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in	

Two-Generation Reproduction Toxicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
occurred at 3 & 30 mg/kg/day across the generations. Reduced serum T4 & T3 and increased TSH were observed in a dose-related manner but were inconsistent across the doses and generations. Thyroid adenomas developed in 2/30 high dose F1males compared to none in controls (Argus 1999, York *et al* 2001).		4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991).	
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic Xenopus laevis tadpoles (Hornung *et al* 2010).	Male PP assay- in rats dosed by gavage, T4 was decreased, TSH was increased, whilst T3 was unaffected at doses from 125 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased from 62.5 mg/kg/day. No other endocrine-related endpoints were affected (Stoker *et al* 2006).	
		Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	

Two-Generation Reproduction Toxicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001).	

Two-Generation Reproduction Toxicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004).	
		Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	

Table 6.5.6.2: Conclusions about Two Generation Reproduction Toxicity Study and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for Perchlorate	**Next Step (as BB)**	**Assay Result for Perchlorate**
Strong evidence that perchlorate has adverse effects on the thyroid gland in rats via a mechanism involving disruption of thyroid hormone synthesis.	If test is to current OECD TG 416 standards, no further testing needed. If not then consider supplemental testing, depending upon existing data.	The existing test is to a high standard and provides evidences of concern with respect to the thyroid gland. However it was conducted to the old TG 416 study design and sensitive endpoints of endocrine disruption *e.g.* sexual maturity, were not measured. It should be noted that (all versions) of TG 416 are not as sensitive as TG 443 with respect to thyroid effects (where TH and developmental neurotoxicity are determined). There is some evidence from developmental neurotoxicity studies that perchlorate has effects on the brain.

Table 6.5.6.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other Considerations (as given in BB)	**Conclusions for Perchlorate**
Sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the ext-1 gen assay provides the most information on endocrine disruption.	Level 5 2-gen assay on perchlorate provides evidences of concern with respect to the thyroid gland only. No developmental and reproductive effects were seen but sensitive endpoints of endocrine disruption *e.g.* sexual maturity, were not measured. There is some evidence from developmental neurotoxicity studies that perchlorate has effects on the brain. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention). A number of endpoints sensitive to disruption of the HPT axis are also included and these provide alerts to possible effects on the developing brain as well as the thyroid. A cohort to include an assessment of the developing nervous system is also part of TG 443.
Effects on apical endpoints may indicate EATS modalities or other mechanisms.	Consideration is valid but there are insufficient data to indicate the presence or absence of other mechanisms.
Consider potency of effects for existing results and whether EATS mechanism is	The magnitude of of the effects is consistent with the dose response relationships of thyroid effects observed in other

Other Considerations (as given in BB)	Conclusions for Perchlorate
credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms.	studies *.e.g.* the effects seen after 90 days of dosing at 10 mg/kg/day
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Perchlorate was given orally in most studies. The available data indicates that it is well absorbed and not metabolized. The existing "*in vivo* effects of concern"do not suggest any ADME issues.

83. Overall conclusions about Two Generation Reproduction Toxicity Study and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence of concern for endocrine activity in mammals, with respect to disruption of thyroid hormones. The possibility of further tests to provide a greater understanding of effects on development, especially in the brain, are prompted by the guidance. The effects seen in mammals also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different.

6.5.7 OECD TG 443: Extended One-Generation Reproductive Toxicity Study (GD No. 150 Section C.4.7)

No data are available

6.6 Perchlorate Case Study Results: *In vitro* Assays that Have Not Yet Completed Validation The Human AR Transcriptional Activation Assay for Detection of Androgen (Ant)agonist-Activity of Chemicals (AR STTA) (GD No. 150 Section Annex 2.1)

No are data available

6.7 Perchlorate Case Study Results: Wildlife *In vivo* Assays That Have Not Yet Completed Validation

6.7.1 Fish (Medaka) Multi-Generation Test (MMGT) (GD No. 150 Section Annex 2.2)

No data are available

6.7.2 Larval amphibian growth and development assay (LAGDA) (GD No. 150 Section Annex 2.3)

No data are available

6.7.3 Avian two generation test (ATGT) (GD No. 150 Section Annex 2.4)

No data are available

6.8 Perchlorate Case Study Results: *In vivo* Mammalian Assays That Have Not Yet Completed Validation Or Not Primarily Designed For Detection Of Endocrine Disruption

6.8.1 Adult male assay (GD No. 150 Section Annex 2.5)

No data are available

6.8.2 TG 408: Repeated Dose 90 Day Oral Toxicity Study in Rodents (GD No. 150 Section Annex 2.6)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay was positive, showing changes in thyroid hormones and alteration of thyroid follicular histology. Existing *in vitro* data show a variety of responses consistent with thyroid interactions (inhibition of iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with disruption of the hypothalamus- pituitary-thyroid axis.

Table 6.8.2.1: Perchlorate data summary

Repeated Dose 90 Day Rodent Oral Toxicity Study data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Perchlorate gave positive results for changes in the thyroid gland, in a non-standard TG 408 90 day assay. Increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) were observed in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters. All effects were reversible after a recovery period of 30 days (Springborn *et al* 1998; Siglin.*et*	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 453- increased thyroid weights in ♂ rats (♀ not studied) after administration in drinking water at 1,300 mg/kg/day. After 40 days thyroid follicular hyperplasia was seen and after 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls 0/22, treated 5/6) (Pajer and Kalisnik 1991).	No data
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).	Male PP assay- in rats dosed by gavage, T4 was decreased, TSH was increased, whilst T3 was unaffected at doses from 125 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased from 62.5 mg/kg/day. No other endocrine-related endpoints were affected (Stoker *et al*	

Repeated Dose 90 Day Rodent Oral Toxicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
al 2000).		2006).	
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic Xenopus laevis tadpoles (Hornung *et al* 2010).	Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	
		TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH levels were observed (Yu *et al* 2000, Caldwell *et al*	

Repeated Dose 90 Day Rodent Oral Toxicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus 1998a; York *et al* 2004, 2006).	

Repeated Dose 90 Day Rodent Oral Toxicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004.	
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	

Table 6.8.2.2: Conclusions about Repeated Dose 90 Day Rodent Oral Toxicity Study and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for Perchlorate	**Next Step (as BB)**	**Assay Result for Perchlorate**
Increased evidence that perchlorate has activity as a thyroid disrupter .	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	Existing 2-gen study: positive for effects on thyroid (Argus 1999; York *et al*, 2001).

Table 6.8.2.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other Considerations (as given in BB)	**Conclusions for Perchlorate**
If existing data are from level 5 then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Level 5 2-gen assay on perchlorate provides evidences of concern with respect to the thyroid gland only. No developmental and reproductive effects were seen but sensitive endpoints of endocrine disruption *e.g.* sexual maturity, were not measured. There is some evidence from developmental neurotoxicity studies that perchlorate has effects on the brain. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention). A number of endpoints sensitive to disruption of the HPT axis are also included and these provide alerts to possible effects on the developing brain as well as the thyroid. A cohort to include an assessment of the developing nervous system is also part of TG 443.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Perchlorate was given orally in most studies. The available data indicates that it is well absorbed and not metabolized. The existing "*in vivo* effects of concern" do not suggest any ADME issues.

84. Overall conclusions about Repeated Dose 90 Day Rodent Oral Toxicity Study and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence of concern for endocrine activity in mammals, with respect to disruption of thyroid hormones. The possibility of further tests to provide a greater understanding of effects on development, especially in the brain, are prompted by the guidance. The effects seen in mammals also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different.

6.8.3 OECD TG 451-3: Combined Chronic Toxicity/Carcinogenicity Studies (GD No. 150 Section Annex 2.7)

Scenario A : Result +, existing *in vitro* data +, existing *in vivo* data +

Scenario A is appropriate because the assay was positive, showing alteration of thyroid follicular histology and increased incidence of thyroid follicular tumours. Existing *in vitro* data show a variety of responses consistent with thyroid interactions (inhibition of iodide uptake and T4 release), and existing *in vivo* data show a variety of endpoints consistent with disruption of the hypothalamus- pituitary-thyroid axis.

Table 6.8.3.1: Perchlorate data summary

Combined Chronic Toxicity/Carcinogenicity Study data for perchlorate	**Existing Results Mechanism (*in vitro* mechanistic data)**	**Existing Results Mammalian Effects (*in vivo* effects of concern)**	**Existing Results Wildlife Effects (*in vivo* effects of concern)**
Perchlorate gave positive results for changes in the thyroid gland, in non-standard TG 453 carcinogenicity assays. Male rats were administered perchlorate in drinking water at 1,300 mg/kg/day (♀ not studied). Thyroid weights weights were increased and thyroid follicular hyperplasia was seen at all timepoints (40, 120, 220 & 730 days). After 2 years benign thyroid tumours were observed in 4/11 treated rats, compared to 0/20 in controls (Kessler and Kruskemper 1966). BALBc mice administered perchlorate in drinking water (2,100 mg/kg/day) developed thyroid epithelial hypertrophy and hyperplasia. Thyroid carcinomas were seen after 46 weeks (controls	Perchlorate inhibited NIS-mediated uptake of iodide in mammalian cell lines (Van Sande *et al* 2003; Agretti *et al* 2011).	TG 408 – increased thyroid weights and histopathological changes (follicular cell hypertrophy and colloid depletion) in rats (♂&♀) after administration in drinking water at 10 mg/kg/day (no changes at 1.0 mg/kg/day. Changes in T4 and TSH occurred at all dose levels (from 0.01 mg/kg/day). No endocrine-related effects observed on reproductive tissues, estrous cycling or sperm parameters (Springborn *et al* 1998; Siglin *et al* 2000).	No data
	Perchlorate inhibited uptake of iodide and reduced incorporation of iodide into thyroglobulin in larval lamprey endostyle tissue (Manzon & Youson 2002).	Male PP assay- in rats dosed by gavage, T4 was decreased, TSH was increased, whilst T3 was unaffected at doses from 125 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased from 62.5 mg/kg/day. No other endocrine-related endpoints were affected (Stoker *et al* 2006).	

Combined Chronic Toxicity/Carcinogenicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
0/22, treated 5/6) (Pajer and Kalisnik 1991).			
	Perchlorate inhibited T4 release from thyroid gland explant cultures from prometamorphic Xenopus laevis tadpoles (Hornung *et al* 2010).	Female PP assay- in rats dosed by gavage, T3 & T4 were decreased from 125 mg/kg/day whilst TSH was increased from 250 mg/kg/day. Thyroid colloid was decreased and follicular cell height was increased. No other endocrine-related endpoints were affected (US EPA 2007, Laws 2011).	
		TG 416- no effects on reproduction or development noted in rats after administration in drinking water at doses from 0.03 to 30 mg/kg/day (endpoints of sexual maturity not determined). Increased thyroid weights and histopathological changes (follicular cell hypertrophy & hyperplasia and colloid depletion) occurred at 3 & 30 mg/kg/day across the generations. Reduced T4 & T3 and increased TSH were observed and 2/30 high dose F1males developed thyroid adenomas (Argus 1999, York *et al* 2001).	
		In short-term studies (up to 14 days), inhibition of thyroidal iodide uptake was observed (Yu *et al* 2000).In drinking water studies up to 22 mg/kg/day, decreased T3 and T4 and increased TSH	

Combined Chronic Toxicity/Carcinogenicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		levels were observed (Yu *et al* 2000, Caldwell *et al* 1995). Increased thyroid weights and decreased follicular lumen occurred (Caldwell *et al* 1995). There was some evidence of adaptation at 1 mg/kg/day as T4 returned to control levels after 14 days (Yu *et al* 2000). An increase in motor activity in pups was determined, after exposure of rats to perchlorate (at doses up to 10 mg/kg/day) during mating and until PND 10 (Bekkedal *et al* 2000). No endocrine-related effects on development were observed when perchlorate was tested in a rat developmental toxicity study (up to 30 mg/kg/day) or a rabbit developmental toxicity study (up to 100 mg/kg/day). Decreases in T4 occurred in rabbit dams but no changes in T3 or TSH (Argus 1998 & 2000; York 2001). In a developmental neurotoxicity study in rats (up to 10 mg/kg/day); brain morphometric changes, increased motor activity, decreased T3 & T4 and thyroid colloid depletion and hyperplasia were reported (Argus	

Combined Chronic Toxicity/Carcinogenicity Study data for perchlorate	Existing Results Mechanism (*in vitro* mechanistic data)	Existing Results Mammalian Effects (*in vivo* effects of concern)	Existing Results Wildlife Effects (*in vivo* effects of concern)
		1998a; York *et al* 2004, 2006). Cross-fostering of pups from rat dams exposed to perchlorate during pregnancy resulted in higher levels of serum perchlorate and lower levels of T4 in pups over the lactational period. T4 levels in pups returned to control values by PND 10 (Mahle *et al* 2003). Pups from dams exposed to perchlorate in drinking water (up to 4 mg/kg/day) had reduced numbers of ovarian antral and preantral follicles (Baldridge *et al* 2004.	
		Perchlorate promoted the carcinogenic activity of DHPN to the thyroid gland Hiasa *et al* 1987).	

Table 6.8.3.2: Conclusions about Combined Chronic Toxicity/Carcinogenicity Study and existing data, and next steps. "Possible conclusions" and "Next step which could be taken to increase evidence if necessary" as given in BB. The result with perchlorate for the suggested assay is shown if available.

Conclusions for Perchlorate	Next Step (as BB)	Assay Result for Perchlorate
Increased evidence that perchlorate has activity as a thyroid disrupter .	Perform assay from level 5 *e.g.* ext-1 or 2-gen assay.	Existing 2-gen study: positive for effects on thyroid (Argus 1999; York *et al*, 2001).

Table 6.8.3.3: Other considerations and conclusions for perchlorate. "Other considerations" are as given in BB. Conclusions for the considerations are based on the data available for perchlorate.

Other Considerations (as given in BB)	Conclusions for Perchlorate
If existing data are from level 5 then there is sufficient information to conclude evidence of concern for endocrine disruption (the ext-1 gen assay provides the most information).	Level 5 2-gen assay on perchlorate provides evidences of concern with respect to the thyroid gland only. No developmental and reproductive effects were seen but sensitive endpoints of endocrine disruption *e.g.* sexual maturity, were not measured. There is some evidence from developmental neurotoxicity studies that perchlorate has effects on the brain. If further testing is required, the new ext-1-gen assay (TG 443) provides the most extensive evaluation of endocrine disruption as it includes many endocrine-sensitive endpoints (*e.g.* AGD and nipple retention). A number of endpoints sensitive to disruption of the HPT axis are also included and these provide alerts to possible effects on the developing brain as well as the thyroid. A cohort to include an assessment of the developing nervous system is also part of TG 443.
Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical	Perchlorate was given orally in most studies. The available data indicates that it is well absorbed and not metabolized. The existing "*in vivo* effects of concern" do not suggest any ADME issues.

85. Overall conclusions about Combined Chronic Toxicity/Carcinogenicity Study and existing data: next steps and other considerations provide a logical course of action to follow. The combined dataset provides evidence of concern for endocrine activity in mammals, with respect to disruption of thyroid hormones. The possibility of further tests to provide a greater understanding of effects on development, especially in the brain, are prompted by the guidance. The effects seen in mammals also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different.

6.8.4 OECD TG 421 Reproduction/Developmental Toxicity Screening Test and TG 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (GD No. 150 Section Annex 2.8)

No data are available

6.9 Perchlorate Case Study: Conclusions

6.9.1 *In vitro* Assays

86. There are no OECD standardized *in vitro* tests for disruption of the thyroid gland and thyroid hormones yet, although they are currently being discussed (OECD, 2006b). In this case study, four *in vitro* studies of perchlorate were identified but as these were not standardized assays they were included as "Mechanism (*in vitro* mechanistic data)". Perchlorate has not been tested in any of the OECD standardized *in vitro* tests included in the guidance document.

6.9.2 *In vivo* Wildlife Assays

87. In general, the advice provided in the GD concerning the results of *in vivo* wildlife screens and tests obtained with perchlorate has proven to be sound, although it should be borne in mind that multi-generation lifecycle data from fish and birds are unavailable, and no full lifecycle data of any description are available for amphibians. The GD advice was relevant both for the negative results obtained in the fish reproductive screening assay (TG 229) and for the positive results in the amphibian metamorphosis assay (TG 231), although in the latter case the outcome can only be evaluated in the light of non-standard partial lifecycle data (which give NOECs as low as 0.027 mg/l). Furthermore, advice applicable to the fish sexual development test (TG 234) and the bird reproduction test (TG 206) also seem appropriate, although in both cases, adverse effects seen in lifecycle or partial-lifecycle tests with these groups generally occur at high mg/l concentrations only. Indeed, the FLCTT data (showing no apical effects on growth or reproduction, although some thyroid histopathology) are a good example of why it can be misleading to rely on the results of a single test – in this case, higher concentrations tested against several other fish species have produced adverse apical impacts.

6.9.3 *In vivo* Mammalian Assays

88. There are data available for perchlorate for CF level 4 and 5 assays: both endocrine specific and general toxicity studies. There are no UT (TG 440), H (TG 441), TG 407, extended 1 generation (TG 443), adult male or TG 421/422 assays available. The endocrine-specific assays conducted on perchlorate are the male and female PP assays. The 90 day repeated-dose toxicity and carcinogenicity studies were not conducted to standard test-guidelines but for the purpose of this case study the protocols were sufficiently similar to TG 408 and TG 453 to be included. The studies used contained sufficient endpoints relevant for the assessment of endocrine activity. A 2-generation study had effects on endpoints of thyroid disruption although sensitive markers of other endocrine modes of action, such as sexual maturation, were not included. As perchlorate affected the thyroid in all these mammalian assays, they were all considered to be positive and therefore only scenario A was tested with perchlorate. In all cases, the guidance provided seemed logical and helpful. The consideration of all the available data (allowing for the restrictions we placed on the case study) allowed us to conclude in every case that further *in vivo* testing for effects on the thyroid was not warranted as the 2-generation assay provided adequate information. However, effects on sexual maturation were not determined and the existing data indicated possible neurotoxicity resulting from developmental effects in the brain. It is acknowledged that these effects are controversial and that discussion of them and the quality of the data showing the effects is outside the remit of this case study. Readers are referred to CEPA (2004) for a summary of the issue. The guidance in this case study, suggested that the extended one-generation assay, including cohorts for developmental neurotoxicity, would be the most appropriate study to assess these endpoints relevant for human health.

6.9.4 Overall Conclusions for Perchlorate

89. In summary, although data on perchlorate from a number of assays are unavailable, this case study suggests that the evaluated building blocks generally provide sound advice about data interpretation and possible next steps. Parts of the case study which evaluated negative assay data (*e.g.* from TG 229 and the FLCTT) provided good examples of the importance of assessing the weight of <u>all</u> available evidence, because much of the wider dataset shows that perchlorate is probably a thyroid disrupter, although at rather high concentrations in groups other than amphibians. In mammals, on the other hand, all of the (*in vivo*) studies demonstrated thyroid disruption. It also seems likely that more restricted 'Existing Data' would not lead to substantially different conclusions, although this was not formally evaluated.

3. Glossary and Abbreviations

ADME	Absorption, distribution, metabolism and excretion
AFSS	Androgenised female stickleback assay
AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
AMA	Amphibian metamorphosis assay
AR	Androgen receptor
AR STTA	Androgen Receptor Stably Transfected Transactivation Assay
ATGT	Avian two generation test
BB	Building Block (as used in OECD GD 150)
CF	Conceptual Framework
CHO cells	Chinese hamster ovary cells
CRH	Corticotrophin-releasing hormone
DHPN	bis(2-hydroxypropyl)nitrosamine
DHT	Dihydrotestosterone
E2	Estradiol
EATS	Estrogenic/androgenic/thyroidogenic/steroidogenic
EC50	Concentration which gives 50% of the maximum effect
ED	Endocrine Disrupter (defined as in GD No. 150). See below for "possible ED". "An ED is an exogenous substance 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." "A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations." (WHO, 2002).
EDTA AG	Endocrine Disruption Testing and Assessment Advisory Group
Endocrine active substance	A substance that affects endocrine endpoints – not necessarily an ED because the effects may not be adverse.
ER	Estrogen Receptor
ER STTA	Estrogen Receptor Stably Transfected Transactivation Assay
Ext -1-gen	Extended-1-generation assay (TG 443) also known as EOGRT Extended-One-Generation Reproductive Toxicity) Study
FETAX	Fetal amphibian assay
FLCTT	Fish life cycle toxicity test

FSDT	Fish sexual development test
FSH	Follicle Stimulating Hormone
GD	Guidance Document
GSI	Gonado-somatic Index
H assay	Hershberger assay
HPT	Hypothalamus-pituitary-thyroid
HPV chemical	High Production Volume chemical
IC50	Concentration at which 50% inhibition is achieved.
i.p.	Intraperitoneal
Ki	The apparent inhibitor dissociation constant
LAGDA	Larval amphibian growth and development assay
LH	Luteinizing hormone
LOEC	Lowest effective concentration
MMGT	Medaka multigeneration test
NIS	Sodium-Iodide-Symporter
NF	Niewkoop and Faber – a staging system for developing amphibians
NMRI	Swiss-type strain of mouse (originally from Naval Medical Research Institute)
NOAEL	No adverse effect level
OECD	Organisation for Economic Cooperation and Development
P	Progesterone
PALM cells	Prostate adenocarcinoma cell line
PND	Post natal day
Possible ED	As defined for the purposes of GD No.150 ”to mean a chemical that is able to alter the functioning of the endocrine system but for which information about possible adverse consequences of that alteration in an intact organism is uncertain.
PP assay	Pubertal/peripubertal assay
PPS	Preputial separation
PR	Progesterone Receptor
RBA	Relative binding affinity
RUCA-I	Endometrial Cell Line
SAT	Sexual accessory tissue
s.c.	Subcutaneous
SD	Sprague-Dawley
SDN-POA	Sexually dimorphic nucleus of the pre-optic area of the brain

STTA	Stably transfected transcriptional activation
T	Testosterone
T3	Triiodothyronine (thyroid hormone)
T4	Thyroxine (thyroid hormone TH)
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin (dioxin)
Tert-OP	4-Tert-octylphenol
TG	Test Guideline
TH	Thyroid hormone (T4)
TSH	Thyroid Stimulating Hormone
USEPA	United States Environmental Protection Agency
UT assay	Uterotrophic assay
VO	Vaginal opening (or patency)
VP	Ventral prostate
VTG	Vitellogenin
YES	Yeast estrogen screen
ZRP	Zona radiata protein
ZPP	Zona pellucida protein

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