Supporting the organisation of a workshop on thyroid disruption –Final Report

Framework Contract ENV.A.3/FRA/2014/0029 on implementation of the Community strategy on Endocrine Disrupters

Brunel University London and DTU National Food Institute Denmark

Written by Brunel University London, Institute of Environment, Health and Societies
National Food Institute, Technical University of Denmark
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Contributors

Professor Andreas Kortenkamp

Dr Olwenn Martin

Dr Alice Baynes

Dr Elisabete Silva

Dr Marta Axelstad

Professor Ulla Hass

EXECUTIVE SUMMARY

This report describes the outcomes of a project that culminated in a workshop on thyroid disruption, held on 29th – 31st March 2017 in Maison-Alfort near Paris, France, on the premises of ANSES.

The workshop objectives were:

- To address and discuss interpretations of experimental laboratory studies, wildlife field data as well as human epidemiological data in relation to the identification of thyroid disrupting substances; and

- To identify ways forward in addressing potential gaps in the test methods in relation to identification of thyroid disrupting substances

As part of this project, case studies were conducted with the intention of elucidating several aspects and issues highlighted as important for understanding thyroid disruption. The case studies covered fipronil, mancozeb, perchlorate, PBDEs/perfluorinated compounds/PCBs and thyroid cancers, and dealt with the following topics (together with the substances chosen to elucidate the topics, listed in italics):

- The **rat as predictor** of human effects – a re-evaluation on the basis of new insights: *perchlorate, thyroid cancers, fipronil case studies*

- The relevance of **compensatory effects** in protecting against thyroid disruption: *perchlorate case study*

- The role of **hepatic metabolism** in thyroid insufficiency: *thyroid cancers, fipronil, PBDE/perfluorinated compounds/PCBs case studies*

- Better measures of **down-stream effects** of thyroid-disrupting chemicals: *mancozeb, PBDE/perfluorinated compounds/PCBs, thyroid cancer case studies*

- The **relevance of thyroid tumours** that arise *via* thyroid hormones (TH) insufficiency: *thyroid cancer case study*

To keep participants abreast of recent developments in thyroid disruptor research, a set of background papers was prepared. These papers focused on the following issues:

- Possible adverse effects in the human following disruption of the hypothalamic-pituitary-thyroid axis

- The thyroid system in fish, amphibians and invertebrates – a comparison with mammalian species

- Interpretation of data from epidemiology, experimental studies and wildlife data for the identification of thyroid disruptors

- Current OECD test methods for the identification of thyroid disruptors, their gaps and possibilities of improvements

During the workshop, three pairs of discussion groups were formed and run in parallel. These groups dealt with the following topics:

- **Discussion Group 1 a,b**: Assays and endpoints for thyroid disruption – *status quo* and perspectives for enhancements

- **Discussion Group 2 a,b**: The human relevance of models of thyroid disruption pathways

- **Discussion Group 3 a,b**: Thyroid systems across taxa: the relevance of mammalian data in environmental assessments and of non-mammalian data in human health assessment

During the course of the workshop it became clear that the interpretation of test results with thyroid disrupting agents is based on an "idealised view" of the thyroid system. According to this view, increases in thyroid stimulating hormone concentrations are expected to occur as a consequence of suppressions of circulating thyroid hormones. The emphasis often placed on measuring circulating thyroid hormones and thyroid stimulating hormone levels is informed by this idea. However, it is difficult to reconcile this view with observations that certain chemicals are capable of inducing thyroid hormone insufficiency without subsequent increases in thyroid stimulating hormone. Sometimes, thyroid stimulating hormone levels even decrease in the wake of thyroid hormone insufficiency. The idealised view also appears incomplete in the light of new evidence of autonomous regulation of thyroid hormone action at the tissue level, without involvement of the hypothalamic pituitary thyroid (HPT) axis and without the corresponding changes in serum thyroid hormones.

The majority of workshop participants concluded that altered circulating levels of thyroid hormones and thyroid stimulating hormone should not be seen as the only markers of thyroid hormone action, or of thyroid disruption. Data sets of serum thyroid hormone measurements contained in many chemical dossiers should be supplemented by data on down-stream effects of an adverse nature, such as effects on the developing brain, or on other TH target organs.

Workshop participants noted the deficiencies of current test guidelines in the OECD Conceptual Framework, and identified the absence of sensitive parameters for the detection of down-stream adverse effects diagnostic of thyroid disruption as a major gap in these guidelines. There was concern that the lack of such parameters hampers the reliable detection of substances as thyroid disruptors.

Detailed suggestions for improvements of the test guidelines were elaborated. Participants highlighted a need to include endpoints for the identification of adverse effects on brain function and brain morphology. The inclusion of endpoints representative of thyroid hormone action at the cellular level, *e.g*. altered expression of thyroid hormone-dependent genes, was also deemed important, as was the implementation of *in vitro* assays that can capture mechanistic aspects of thyroid disruption.

A strategy for improving test guidelines could unfold at three levels, by improving thyroid histology, by extending exposure periods to windows of sensitivity during development, and by incorporating downstream effects diagnostic of brain morphology and function, lipid metabolism and the cardiovascular system.

Until these gaps are filled, it is necessary to evaluate substances on the basis of incomplete data on thyroid disruption. Following considerable discussion, all groups came to a consensus regarding the interpretation of changes in serum thyroxine (T4) levels in rodents. If such changes occur, for any reason, including altered hepatic clearance, they should be considered a thyroid effect, unless the mechanism of altered clearance (*i.e*. the specific enzymes or pathways impacted) can be determined not to be relevant to humans or wildlife of concern. Such findings should be regarded as relevant for regulatory decisions unless, on the basis of further compound specific information, it is possible to exclude the human relevance of such effects.

Observations of thyroid disruption in rodent laboratory studies can be useful for the identification of thyroid disrupting properties in other wildlife mammalian species. Considering the preservation of the thyroid system across taxa, such data would also

raise concerns for *e.g*. birds, fish or amphibians, although species-species extrapolations will not be straight-forward due to differences in exposure routes (*e.g*. dermal in amphibians *versus* oral in rodents) and other factors (*e.g*. the presence of the placenta in mammals).

Conversely, data from non-mammalian test species can be used to inform on the mode of action of putative thyroid disrupting chemicals in mammals. This is strongly supported by the observation that most amphibian thyroid disruptors have elicited positive responses also in rodent tests.

List of abbreviations

ADHD Attention-deficit hyperactivity disorder

ADI Acceptable Daily Intake

AhR Aryl hydrocarbon Receptor

ANSES Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

AOP Adverse Outcome Pathways

BDNF Brain-derived neurotrophic factor

BTEB Basic transcription element binding protein

CAR Constitutive androstane receptor

CHO Chinese Hamster Ovary

CI Confidence Interval

CYP P450 Cytochrome P 450

D1, D2 D3 Iodothyronine deiodinase types 1, 2, 3

DAR Draft Assessment Report

DEHP Diethyl-hexyl phthalate

DNT Developmental Neurotoxicity

ECHA European Chemicals Agency

ED Endocrine Disruption

EDC Endocrine Disrupting Chemical

EDTA AG Endocrine Disrupters Testing and Assessment Advisory Group

EDSP Endocrine Disrupter Screening Programme (USA)

EFSA European Food Safety Authority

EOGRTS Extended One Generation Reproductive Toxicity Study

EPA Environmental Protection Agency

ETU Ethylenethiourea

EU European Union

FDA Food and Drug Administration

FTC Follicular Thyroid Cancer

GABA Gamma Amino Butyric Acid

HPT Hypothalamic Pituitary Thyroid axis

IARC International Agency for Research on Cancer

IGF1 Insulin-like growth factor 1

LOAEL Lowest Observed Adverse Effect Level

MCT Mono Carboxylate Transporters

MIE Molecular Initiating Event

NHANES National Health and Nutrition Examination Survey

NIS Sodium Iodine Symporter

NTCP Na Taurocholate co-transporting polypeptide

NOAEL No Observed Adverse Effect Level

NOEC No Observed Effect Concentration

NTP National Toxicology Programme (USA)

OECD Organisation for Economic Cooperation and Development

PAH Polycyclic aromatic hydrocarbons

PBDE Polybrominated diphenyl ethers

PCB Polychlorinated biphenyls

PFAA Perfluoro alkyl acids

PFOA Perfluoro octanoic acid

PFOS Perfluoro octanoic sulfonic acid

PND Postnatal Day

PTC Papillary Thyroid Cancer

PTU Propylthiouracil

PXR Pregnane X receptor

SD Sprague Dawley

TBG Thyroxine Binding Globulin

T3 Triiodothyronine

T4 Thyroxine

TH Thyroid hormones (includes T3 and T4)

TPO Thyroid PerOxidase

TR Thyroid hormone Receptor

TSH Thyroid Stimulating Hormone

TSHR Thyroid Stimulating Hormone Receptor

TTR Transthyretin

UDP Uridine diphosphate

US EPA United States Environmental Protection Agency

Contents

1. INTRODUCTION

This document is the Final Report for the project **Supporting the organisation of a workshop on thyroid disruption**.

The objective of this project was to provide technical and administrative support for the preparation, execution and follow-up of a workshop on thyroid disruption. The workshop objectives were:

- To address and discuss interpretations of experimental laboratory studies, wildlife field data as well as human epidemiological data in relation to the identification of thyroid disrupting substances; and

- To identify ways forward in addressing potential gaps in the test methods in relation to identification of thyroid disrupting substances

In line with the technical specifications for this service request, and on the basis of the above specific objectives, the study included the following tasks:

Task 1. Set up a Scientific Expert Group which provides scientific and technical guidance on the drafting of the background and discussion papers for the workshop and on its design, planning and organisation.

Task 2. Design and plan a workshop on thyroid disruption and develop and draft background and discussion papers to be used to provide a basis for the deliberations of workshop participants at the workshop.

Task 3. Conduct the workshop on thyroid disruption.

Contents of this report

This report describes all the results obtained in relation to all tasks of the project, under the following headings:

- The Scientific Expert Group and the Steering Group
- The workshop concept
- Workshop venue and programme
- Case studies of thyroid disrupting chemicals
- Additional background papers for the workshop
- Background and topic outlines for the workshop discussion groups
- Workshop report
- Conclusions

2. THE SCIENTIFIC EXPERT GROUP AND STEERING GROUP

To secure scientific guidance from recognised scientists with expertise in the thyroid disruption area, a Scientific Expert Group was set up. The role of this group was in commenting on the workshop concept, on the content of the background and discussions documents that were to be drafted to prepare participants for the workshop, and on general aspects of workshop organisation. Furthermore, members of the scientific expert group were tasked with moderating the discussion groups that were formed from workshop participants.

The following experts served on the scientific expert group for this project:

- Dr Niklas Andersson, ECHA, Helsinki, Finland
- Dr Marie-Noelle Blaude, Scientific Institute of Public Health WIV-ISP, Brussels, Belgium
- Dr Patience Browne, OECD, Paris, France
- Professor Nicolas Chevalier, Centre Hospitalier Universitaire de Nice, Nice, France
- Professor Barbara Demeneix, Muséum National d'Histoire Naturelle, Paris
- Dr Margareta Halin Lejonklou, KEMI, Stockholm, Sweden
- Dr Timo Hamers, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands
- Dr Marie-Louise Holmer, Danish Environment Ministry, Copenhagen, Denmark
- Professor Josef Koehrle, Charite Hospital, Berlin, Germany
- Dr Martine Röhl, FPS Public Health, Brussels, Belgium
- Dr Catherine Viguie, INRA, Toulouse, France
- Dr Gro Dehli Villanger, Norwegian Institute of Public Health, Oslo, Norway
- Professor Tom R Zoeller, University of Massachusetts, Amherst, USA

The Steering Group for this project had responsibility for coordinating the implementation of this project. It was composed of:

- Dr Peter Korytar, European Commission, DG ENV
- Maiken Guldborg Rasmussen, European Commission, DG ENV
- Dr Sharon Munn, European Commission, DG JRC
- Dr Elise Grignard, European Commission, DG JRC
- Dr Cecile Michel, ANSES
- Dr Claire Beausoleil, ANSES

3. THE WORKSHOP CONCEPT

The task was to organise a scientific workshop on thyroid disruption. In discussions with the Scientific Expert Group and the Steering Group the following **specific workshop aims** were agreed upon:

- To discuss issues related to the **interpretation** of thyroid disruption test outcomes
- To identify **gaps** in testing guidelines
- To elaborate **recommendations** of how to fill these gaps
- To reach a **consensus** on interpretation and testing issues (if possible)

The workshop was to be held over three days on the premises of ANSES in Alfortville.

The programme was to comprise formal presentations on key aspects of thyroid disruption, with the aim of providing up-to-date overviews of relevant aspects of the topic, as well as discussion groups composed of workshop participants. The discussion groups were to deal with specific aspects of thyroid disruption, identify the contours of diverging scientific opinions (if any) and, if possible, arrive at common recommendations and suggestions.

Three discussion topics were identified:

- **Topic 1**: Assays and endpoints for thyroid disruption – status quo and perspectives for enhancements
- **Topic 2**: The human relevance of models of thyroid disruption pathways
- **Topic 3**: Thyroid systems across taxa: the relevance of mammalian data in environmental assessments and of non-mammalian data in human health assessment

To arrive at manageable group sizes, it was decided to set up two groups for each topic, here designated a and b, to be run in parallel. To ensure continuity of the groups' discussion process, workshop participants remained with their chosen group for the duration of the workshop.

Each group was moderated by two members of the Scientific Expert Group, and assisted by one member of the Study Team who acted as rapporteur and note taker. The discussion groups with their moderators and rapporteurs are shown in the workshop programme below.

4. WORKSHOP VENUE AND PROGRAMME

ANSES - Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail – kindly agreed to host the workshop on their premises at:

14 rue Pierre et Marie Curie

94701 Maison-Alfort Cedex

The workshop began on **29 March 2017** at 11:00 and closed on **31 March 2017** at 15:00.

The workshop programme is shown below.

Wednesday 29th March	
10:30	Coffee and registration
11:00	**Welcome**: *Peter Korytar (DG ENV), Roger Genet (Director General of ANSES)*
11:15	**Introduction to the workshop: aims, structure and logistics** *Andreas Kortenkamp (Brunel University London)*
11:30	**Keynote lecture: thyroid disruptor research in the 21st century** *Tom Zoeller (UMass Amherst, USA), Barbara Demeneix (National Museum of Natural History, Paris), Josef Köhrle (Charite Berlin), Catherine Viguie (INRA Toulouse)*
12:30	**Summary of pertinent aspects of case studies of thyroid disruption** *Andreas Kortenkamp, Ulla Hass, Olwenn Martin, Marta Axelstad (Brunel University London)*
13:00	Lunch
14:00	**Overview of test guidelines for thyroid disruption** *Patience Browne (OECD)*
14:30	**New insights from eco-toxicological animal models (fish, amphibians) - implications for testing strategies** *Alice Baynes (Brunel University London)*
15:00	**Substances interacting with the thyroid hormone synthesis might trigger adverse effects on the HPT and HPG axes – a case study on perchlorates from the ecotoxicological perspective** *Eva Fetter (Umweltbundesamt, Germany)*
15:30	Coffee
16:00	**Pathways of thyroid disruption and their relevance to human risk assessment – insights from a case study with fipronil** *Marta Axelstad (DTU)*
16:30	**Discussion groups (DG) begin work in separate parallel sessions (two groups, a and b, deal with the same topic)**

	DG 1 a b: Assays and endpoints for thyroid disruption – status quo and perspectives for enhancements *Moderators:* *DG 1a – Tom Zoeller (UMass Amherst), Patience Browne (OECD)* *DG 1b – Barbara Demeneix (National Museum of Natural History, Paris), Niklas Andersson (ECHA)* **DG 2 a b: The human relevance of models of thyroid disruption pathways** *Moderators:* *DG 2a – Timo Hamers (VU University of Amsterdam), Marie-Noelle Blaude (Scientific Institute of Public Health, Belgium)* *DG 2b – Josef Koehrle (Charite Berlin), Gro Dehli Vilanger (Norwegian Institute of Public Health)* **DG 3 a b: Thyroid systems across taxa: the relevance of mammalian data in environmental assessments and of non-mammalian data in human health assessment** *DG 3a – Margareta Lejonklou (KEMI Sweden), Catherine Viguie (INRA Toulouse)* *DG 3b – Marie-Louise Holmer (Danish Environmental Protection Agency), Martine Röhl (FPS Public Health)*
17:45	**Working groups briefly report back to plenary**
18:30	Welcome drinks

Thursday 30th March	
8:30	Coffee
9:00	**Hepatic metabolism and thyroid disruption - an example from REACH dossier evaluations** *Niklas Andersson (ECHA)*
9:30	**Enigmatic effects patterns and down-stream TH effects - lessons from the PBDE and mancozeb case studies** *Olwenn Martin (Brunel University London), Marta Axelstad (DTU)*
10:00	**The role of TSH elevation in thyroid cancers: Evidence from human studies and rodent tumour models** *Andreas Kortenkamp (Brunel University London)*
10:30	Coffee
11:00	**Thyroid disruption: Insights from human epidemiology** *Andreas Kortenkamp (Brunel University London)*
11:30	**Discussion groups resume in separate sessions**

13:00	Lunch
14:00	**Discussion groups continue in separate sessions**
15:30	Coffee
16:00	**Discussion groups present the status of their deliberations for a first plenary discussion**
17:00	Close
19:00	Workshop dinner

Friday 31st March	
8:30	Coffee
9:00	**Discussion groups resume and prepare their conclusions for debate in the plenary session**
10:00	**DG 1 a b: Assays and endpoints for thyroid disruption – status quo and perspectives for enhancements** **Presentation to plenary and discussion**
11:00	Coffee
11:30	**DG 2 a b: The human relevance of models of thyroid disruption pathways** **Presentation to plenary and discussion**
12:30	Lunch
13:30	**DG 3 a b: Thyroid systems across taxa: the relevance of mammalian data in environmental assessments and of non-mammalian data in human health assessment** **Presentation to plenary and discussion**
14:30	**General discussion and wrap-up** *Andreas Kortenkamp (Brunel University London)*
15:00	**Close** *Peter Korytar (DG ENV), Cécile Michel (ANSES)*

5. CASE STUDIES OF THYROID DISRUPTING CHEMICALS

The technical specifications of this project stipulated that a number of case studies of thyroid disrupting chemicals were to be conducted and supplied to workshop participants as material to enable effective preparation for the workshop. In discussions with the Scientific Expert Group and the Steering Group it was agreed that the case studies should focus on the following aspects and topics, all of which were deemed to be of relevance for the understanding of thyroid disruption:

- The **rat as predictor** of human effects – a re-evaluation on the basis of new insights
- The relevance of **compensatory effects** in protecting against thyroid disruption
- The role of **hepatic metabolism** in thyroid insufficiency
- Better measures of **down-stream effects** of thyroid-disrupting chemicals
- The **relevance of thyroid tumours** that arise via thyroid hormones (TH) insufficiency

Based on suggestions by the Study Team, the following substances were selected for these case studies:

- Fipronil
- Mancozeb
- Perchlorate
- PBDEs/perfluorinated compounds/PCBs

It was also decided to make thyroid cancers and the role of thyroid stimulating hormone (TSH) in its genesis the topic of a separate case study.

The substances selected for the case studies covered several of the aspects and issues highlighted as important for understanding thyroid disruption:

- The **rat as predictor** of human effects – a re-evaluation on the basis of new insights: *perchlorate, thyroid cancers, fipronil case studies*
- The relevance of **compensatory effects** in protecting against thyroid disruption: *perchlorate case study*
- The role of **hepatic metabolism** in thyroid insufficiency: *thyroid cancers, fipronil, PBDE/perfluorinated compounds/PCBs case studies*
- Better measures of **down-stream effects** of thyroid-disrupting chemicals: *mancozeb, PBDE/perfluorinated compounds/PCBs, thyroid cancer case studies*
- The **relevance of thyroid tumours** that arise via TH insufficiency: *thyroid cancer case study*

The case studies of fipronil, mancozeb, perchlorates and PBDEs/perfluorinated compounds/PCBs follow a common structure with the following headings:

- Summary
- Scope and aims of the case study
- The substance and its uses

- Observations from human studies with relevance to thyroid disruption
- Observations from experimental studies

Due to the nature of the subject matter, the case study on thyroid cancers and the role of thyroid hormone insufficiency somewhat deviates from this scheme.

5.1 FIPRONIL CASE STUDY

5.1.1 Summary

The fipronil case study can be used as a starting point for exploring and discussing thyroid disrupting compounds that increase thyroid hormone (TH) clearance, via induction of liver enzymes, and the human relevance of this molecular initiating event (MIE).

The effects of the insecticide fipronil on the thyroid hormone system have been investigated in a substantial number of repeated-dose toxicity studies. Results from these studies have been included in the present case study based on study summaries from the EFSA Draft Assessment Report (DAR) (2004) and on peer reviewed papers found in the open literature. From all these studies, the same picture emerges, namely that in rats fipronil causes marked decreases in circulating levels of thyroxine (T4), even at very low oral doses (0.02 & 0.06 mg/kg/day). Increased TSH levels, increased thyroid gland weights and thyroid follicle hypertrophy have been consistently found at somewhat higher exposure levels (1.5 and 13-16 mg/kg/day) (DAR 2004, Leghait et al 2009). A statistically significant increase in incidences of follicular cell tumours has been observed in an oncogenicity study in rats (13-16 mg/kg/day) (DAR 2004). The mechanism behind the adverse effects on the thyroid hormone system is likely via induction of liver enzymes that contribute to increased TH clearance, since increases in the activity and mRNA expression of two types of phase II hepatic enzymes, the uridine 5'-diphospho (UDP)-glucuronosyltransferases (UGT) and the sulfotransferases (SULT) have been observed after fipronil exposure (DAR 2004, Leghait et al. 2009, Roques et al 2012). The main metabolic pathway in the liver, leading to formation of the active metabolite, fipronil sulfone, is mediated by cytochrome P450 (CYP) enzymes, and is at least partly controlled by the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Roques et al. 2013).

Repeated-dose toxicity studies on fipronil have also been performed in mice, dogs and sheep. A study by Ferreira et al (2012) indicated that mice exposed to fipronil showed histopathological alterations in their thyroid tissues. In contrast, no thyroid toxicity was observed in a life-time cancer study in mice, and no thyroid toxicity has been seen in subchronic repeated dose toxicity studies in dogs (DAR 2004). In toxicity studies performed in sheep, an animal model more closely resembling human thyroid physiology, moderate alterations in free thyroxine (T4) clearance have been observed, but this did not lead to any adverse effects on thyroid hormone levels (Leghait et al 2010). The reason for the species difference between sheep and rats could be that in sheep the concentrations of the active metabolite (fipronil sulfone) in plasma were much lower compared to rats (Leghait et al 2010).

One aim of the present case study is to discuss the relevance of the rat as a model for assessing the risk of chemically induced thyroid disruption in humans, focusing on compounds which induce liver-mediated increase in TH clearance. Throughout the case study, several questions related to this subject are therefore discussed. Should chemically induced adverse effects in the rat thyroid system be viewed as relevant to humans? Are such alterations a sign of endocrine disruption, if they are mediated via the liver? Is this mode of action occurring in humans? And are statistically significant reductions in circulating T4 level in rats toxicologically relevant, in the absence of effects on other thyroid endpoints?

At the end of the present case study data from fipronil studies have also been compared to data from rats exposed to the biocide triclosan. Like fipronil, triclosan exposure decreases circulating T4 levels by induction of liver enzymes, via CAR/PXR. Until recently this MIE appeared to be the only relevant one for triclosan, however new research has shown that triclosan also binds to the carrier protein transthyretin (TTR) (Weiss et al 2015). This binding could be an additional explanation for the observed decrease of free circulating T4 caused by triclosan. In contrast to fipronil, triclosan did not affect TSH levels in any of the performed studies. The implications of this difference contribute with an additional point of discussion in the present case study.

5.1.2 Scope and aims of this case study

The aspect of thyroid disruption which will be discussed, based on the present case study, is the relevance of the rat as a model for humans in the context of thyroid disruption caused by liver enzyme induction. More specifically, the following questions will be addressed, both in relation to their scientific implications and their regulatory significance and impact:

- Are chemically induced adverse effects to the liver, and hence the thyroid gland, in rats relevant to humans? Such effects have in "classical toxicology" often been dismissed as irrelevant, since rats (due to species differences in T4 half- lives and in carrier proteins) have been viewed as particularly sensitive to disturbances in their thyroid hormone system. However, more recent research has shown that half lives of the active hormone (T3) are much more similar in both species, and that TTR is the main physiologically relevant distributor in humans, perhaps making the "classical"arguments outdated?

- Are effects on the hypothalamic-pituitary-thyroid (HPT) axis in rats (e.g. increased TSH or altered thyroid gland morphology) signs of endocrine disruption if they are attributed to a liver-mediated mechanism? And is this mode of action relevant when discussing endocrine disruption in humans?

- Are statistically significant reductions in circulating T4 level in rats toxicologically relevant, in the absence of effects on TSH and thyroid gland weight and histopathology, and can/should significant T4 decreases in themselves be used for setting NOAEL/LOAEL values?

In order to address these discussion points, the present case study includes information from fipronil studies found in the open literature as well as data from the EFSA Draft Assessment Report for Fipronil (DAR 2004). However, the presented case is not meant to be an exhaustive substance review, so only data relevant to the above mentioned specific discussion points is presented here.

In order to properly address the last bullet point, data from Fipronil is compared and contrasted to data from the biocide triclosan, which also causes decreases in circulating T4 levels, but where no other apparent adverse effects on the HPT-axis are seen.

Data from a developmental neurotoxicity study (US EPA guideline 1991) on Fipronil are also presented in the EFSA DAR (2004). These data showed that fipronil-exposed hypothyroxinemic dams gave birth to offspring which did not show deficits in motor activity or learning behaviour, as would have been expected after developmental hypothyroidism. These results will be discussed in the case study on Mancozeb, where the consequences of maternal TH insufficiency for the offspring will be discussed.

5.1.3 Fipronil and its uses

Fipronil (fluocyanobenpyrazole) is a widely used, second-generation phenilpirazol insecticide that is used in agriculture and veterinary medicine for protection against fleas, ticks, ants, cockroaches and other pests. The insecticide blocks the chloride channels associated with gamma-amino butyric acid (GABA) and glutamate (Glu) receptors in insects (Magalhães *et al* 2015*)*.

5.1.4 Observations from human studies with relevance to thyroid disruption

The only epidemiology study that has investigated the associations between fipronil exposure and thyroid function in humans is that by Herin et al. (2011). The primary objective of the study was to test the hypothesis that chronic occupational fipronil exposure could be associated with abnormal thyroid function. In this study, 159 workers of a factory manufacturing fipronil-containing veterinary drugs were assessed, and serum concentrations of TSH, total thyroxine, free thyroxine, fipronil, and fipronil sulfone were measured. Positive and significant correlations were observed between serum fipronil levels or levels of the main metabolite, fipronil sulfone, and duration of fipronil exposure. No significant increases in thyroid function test abnormalities were seen, with increased exposures, but serum fipronil sulfone concentrations were negatively correlated with TSH concentrations. This is a pattern opposite to what is seen in rat studies, where TSH levels increase after fipronil exposure, which raises the possibility that in humans, fipronil could have a central inhibitory effect on TSH secretion. Larger epidemiological studies would be desirable in order to confirm and elaborate on these findings.

5.1.5 Observations from experimental studies

5.1.5.1 Toxicity studies performed in rats

In the EFSA DAR on fiprionil (2004), results not published in the open literature from a substantial number of repeated-dose toxicity studies in rats investigating thyroid disruption are presented. For some of the study results, tables are available in the DAR, whereas other results are concluded upon, but not shown. In all of the performed studies (28-day, 90-day, 2 year and 2-generation studies) oral fipronil exposures caused decreases in circulating levels of thyroxine (T4) in rats. Even at very low doses (0.02 & 0.06 mg/kg/day) statistically significant decreases in T4 levels were seen, whereas increased TSH levels, increased thyroid gland weights and thyroid follicle hypertrophy were consistently found at somewhat higher exposure levels (1.5 mg/kg/day and above, in repeated dose-, oncogenicity- and 2-generation study) (DAR 2004). Generally, the doses required to induce adverse effects on the thyroid hormone system decreased with prolonged exposures to fipronil. In the oncogenicity study in rats, exposure to 13-16 mg/kg/day also significantly increased incidences of follicular cell tumors in both males and females (DAR 2004).

These unpublished studies have been complemented recently by studies available in the open literature. In 2009, Leghait et al published the first such study investigating the effects of fipronil exposure in rats, with the aim of evaluating the effects of fipronil on thyroid hormone (TH) concentrations and elimination. In thyroid-intact female rats, fipronil treatment at oral doses of 3 mg/kg bw/day for 14 and 28 days decreased both total and free TH plasma concentrations. Accordingly, thyroid stimulating hormone (TSH) plasma levels increased. Plasma concentrations of fipronil's main metabolite, fipronil sulfone, were at least 20-fold higher than those of fipronil. Thyroidectomized + T3 supplemented (euthyroid-like) rats were hereafter used to evaluate both total and free T4 clearances. This study showed that fipronil treatment induced a twofold increase in total and free T4 clearances. The treatment was also associated with an increase in hepatic microsomal 4-nitrophenol UDP-glucuronosyltransferase activity involved in T4 glucuronidation. Thus, fipronil-induced thyroid disruption was likely mediated by increased hepatic enzyme activity (Leghait et al 2009).

The same research group continued research in this area and investigated whether fipronil biotransformation into fipronil sulfone by hepatic cytochromes P450 (CYP) could act as a potential thyroid disruptor. The aim of this study was to determine if fipronil sulfone treatment could reproduce the fipronil treatment effects on T4 clearance and CYP induction in rats. The study showed that both fipronil and fipronil sulfone treatments increased total and free T4 clearances to the same extent, using THX + T3, euthyroid-like rats. Both treatments induced a 2.5-fold increase in Ugt1a1 and Sult1b1 messenger RNA (mRNA) expressions and a twofold increase in UGT1A activity suggesting that T4 elimination was mediated, at least in part, by hepatic uridine 5'-diphospho-

glucuronosyltransferases (UGT) and/or sulfotransferases (SULT) induction. Both treatments induced a 10-fold increase in Cyp3a1 and Cyp2b2 mRNA expressions, concomitant with a threefold increase in CYP3A immunoreactivity and a 1.7-fold increase in antipyrine clearance, a biomarker of CYP3A activity. All these results showed that fipronil sulfone treatment could reproduce the fipronil treatment effects on T4 clearance and hepatic enzyme induction in rats. The authors concluded that the potential of fipronil sulfone to act as a thyroid disruptor was all the more critical, because it persists much longer in the organism than fipronil itself (Roques et al 2012).

Investigations of the pathways involved in fipronil-induced liver gene expression regulations have continued by hepatic gene expression studies of rats treated with fipronil or vehicle. Fipronil treatment led to the upregulation of several genes involved in the metabolism of xenobiotics, including the cytochrome P450 Cyp2b1, Cyp2b2 and Cyp3a1, the carboxylesterases Ces2 and Ces6, the phase II enzymes Ugt1a1, Sult1b1 and Gsta2, and the membrane transporters Abcc2, Abcc3, Abcg5, Abcg8, Slco1a1 and Slco1a4. Based on the large overlap with the target genes of constitutive androstane receptor (CAR) and pregnane X receptor (PXR), the authors postulated that these two nuclear receptors are involved in mediating the effects of fipronil on liver gene expression in rodents (Roques et al 2013).

Recently Magalhaes et al (2015) tested the effects of fipronil in pregnant rat dams. A commercial product containing fipronil (0.1, 1 and 10 mg/kg/day) was administered orally to pregnant Wistar rats (n=10) from the 6th to the 20th day of gestation. The authors found an increase in maternal aggressive behaviour on PND 6, and concluded this to be mediated through effects on GABA(A) receptors, but saw no adverse effects on maternal thyroid gland histopathology (Magalhaes et al 2015). Unfortunately, thyroid hormone levels were not assessed. Since several previous studies in rats have shown fipronil to adversely affect thyroid gland weight and histopathology in rats at similar dose levels, it is most likely the relatively short time of exposure (2 weeks) chosen for this study explains why no adverse effects on thyroid histopathology were seen.

5.1.5.2 Toxicity studies performed in other species

In 2010, Leghait et al. performed a study in sheep. This species is more similar to humans compared to the rat, as far as thyroid carrier proteins are concerned. Sheep express thyroid binding globulin (TBG) at levels similar to those in humans and, unlike in the rat, this expression is not developmentally regulated. Moreover, ovine TBG has pharmacological properties, such as T4 maximal binding capacity and affinity, close to those of human TBG. Since however new data suggests that TTR is the more relevant carrier protein in humans (Alshehri et al 2015), the species similarities between human and sheep may from a toxicological point of view be less important than first assumed.

In the study in thyroidectomised T3-treated euthyroid like rams, a dose of 5 mg/kg of fipronil every 4 days for 11 weeks did not modify secretory profiles of TSH, tT3 and tT4 and only a moderate effect on free T4 clearance was observed. According to the authors, one reason for this marked species difference between rats and sheep could be the differences in internal exposures to the active metabolite fipronil sulfone (Leghait et al 2010), which is responsible for the adverse effects on the rat thyroid hormone system (Roques et al 2012). In rats which were continuously dosed with fipronil, the fipronil sulfone/fipronil ratio at steady state was higher than 40 (Leghait et al 2010), whereas in sheep this ratio was below 1 for the first 24 hours following administration, and fluctuated between 3 and 6 at steady state . This means that in sheep the concentrations of the active molecule may not have been high enough to induce adverse effects on the thyroid hormone system (Leghait et al 2010). In the Fipronil DAR (DAR 2004) repeated dose toxicity studies in dogs and mice are also reported. In one of the studies performed in beagle dogs (n=2/sex) exposed to 1, 10 or 20 mg/kg /day for 3 weeks, T3, T4 and TSH levels were reported not to be significantly affected (though these data were not actually shown in the DAR report). Furthermore, after 6 weeks of treatment no effect on thyroid histopathology was evident (these data were not shown either). The highest dose in this study caused signs of systemic toxicity (weight loss) and the two highest doses caused adverse effects in the neurological examinations, indicating that in dogs adverse

neurological effects of fipronil seem to be the critical endpoint. A 90-day toxicity study and two 1-year toxicity studies in dogs were also summarized in the DAR. These studies focused on examining adverse neurological effects of fipronil, and there was no mentioning of TH levels, thyroid weights or –histopathology. It is therefore difficult to determine whether these endpoints were not affected or simply not assessed in the latter dog studies.

Whether thyroid gland weights and histopathology was investigated in a carcinogenicity study performed in mice is also somewhat difficult to assess, based on the information presented in the DAR. In this study, adult mice (n=20/sex) were exposed to low doses of fipronil (0.01 – 3.5 mg/kg bw/day) for 78 weeks. No adverse effects on thyroid weights or histopathology were mentioned in the DAR. Whether thyroid endpoints were actually investigated in the performed mouse study, is somewhat unclear, since the methods section in the DAR only stated that "selected organs were weighed and a comprehensive range of tissues preserved". But as the study was a carcinogenicity study performed according to a USEPA guideline (1984), it seems reasonable to assume that thyroid tissue was assessed and found not to be affected by the tested doses of fipronil. If this is indeed the case, this indicates a clear species difference in sensitivity of thyroid effects between mice and rats for this compound. Ferreira et al (2012) have published a study in which female mice (n=5) received IP injections with fipronil for 7 days, at doses of 15, 25 and 50 mg/kg bw/day. In this study the authors only investigated thyroid histopathology, and the publication unfortunately did not state whether these relatively high doses of fipronil caused any signs of systemic toxicity. It furthermore seems that the authors did not perform any statistical analysis of the data, but simply compared the histological appearance of slides from different exposure group. The results from this study are therefore not very reliable, but if indeed correct, they indicate that high doses of fironil could cause alterations in the thyroid tissue in mice, with follicular disorganization and decreased size of most follicles. The authors further claimed to have shown that the action of fipronil not only caused disorganization in the thyroid tissue, but also altered the chemical composition of the colloid itself (Ferreira et al 2012). These results will have to be repeated in a larger and better designed study, in order to assess whether histopathology of mouse thyroid glands is indeed affected by fipronil exposure.

Roques et al (2013) investigated whether the fipronil-induced changes in liver gene expression seen in rats could be reproduced in mice, and further studied the effects of fipronil in wild-type, CAR- and PXR-deficient mice. They found that fipronil treatment and the subsequent induction of CAR and PXR thyroid-related target genes in mouse liver was not associated with a marked increase of total T4 clearance, as observed in rat (Roques et al 2013). In the CAR and PXR deficient mice, for most of the genes studied, gene expression modulations were abolished in the liver of PXR-deficient mice, but were only reduced in CAR-deficient mice, indicating that PXR may be more involved in mediating the effect of fipronil on liver gene expression than CAR (Roques et al 2013). Previous studies have shown that other liver enzyme inducers which activate CAR and PXR, also have smaller effects on UDP-glucuronosyltransferase activity in mice compared to rats (Viollon-Abadie et al 1999). Thus, different regulation of phase II glucuronidation enzymes in mice and rats may well contribute to the different sensitivities of these species, in regards to compounds causing increased thyroid hormone clearance such as fipronil (Roques et al 2013).

Taken together, the results of thyroid disruption in other species than rats do indicate that fipronil-induced thyroid hormone clearance seems to be much more marked in rats, than in dogs, sheep or mice. However, for several of the (non-rat) studies presented in the DAR, it was difficult to determine whether the thyroid gland can be ruled out as the target organ, or whether this was just not properly assessed. Fipronil sulfone is the main fipronil metabolite in rats, sheep and humans (Leghait et al 2010). However, the rate of fipronil sulfone formation is about fourfold higher in rat liver microsomes than in human ones (Tang et al., 2004), and accidental human exposure to fipronil has indicated that the ratio of fipronil sulfone/fipronil plasma concentrations at 24 h post-ingestion is about 0.25–0.5 (Mohamed et al., 2004). This quantitative difference in fipronil metabolism

could by some be interpreted as a reason not to use the results from toxicity studies of fipronil performed in rats for human risk assessment. However, given the long half-life of firponil sulfone, it is very likely that after long term exposure in humans, the internal exposures of fipronil sulfone will be higher than those of the parent compound itself. Therefore human internal exposures have to be thought of in terms of exposure to the metabolite, and the fact that this fipronil sulfone is more efficiently and rapidly produced in the rat compared to humans and sheep, does not change the overall possibility of adverse effects occurring.

Another point which is evident from the present case study is the importance of toxicokinetic data of compounds under investigation for thyroid disruption. In chemical registration dossiers this information is sometimes present from rat models, but often not available from any other species. Such information can however be very useful or even necessary when deciding how relevant an animal model is in relation to human risk assessment.

5.1.5.3 In vitro and mechanistic studies

Fipronil and fipronil sulfone have also been investigated for endocrine disrupting mechanisms in vitro. In a thyroid hormone receptor (TRβ) assay, only fipronil sulfone showed anti-thyroid hormone activity with a RIC_{20} of 8.2 × 10(-7)M[1]. Furthermore, molecular docking was employed to support the results in TR assay with lower MolDock score for fipronil sulfone (Lu et al 2015).

Mitchell et al (2016) investigated transcript levels in primary human hepatocytes exposed to fipronil. Fipronil exposure was very effective in eliciting changes, as RNA-Seq showed that fipronil at 10 µM increased the levels of 2246 transcripts and decreased the levels for 1428 transcripts (Fipronil was 21-times more effective than the insect repellent DEET (N,N-diethyl-m-toluamide) even though the treatment concentration was 10-fold lower). The data indicated that fipronil can regulate the gene expression of several classes of phase I, II and III enzymes/ transporters some of which are potentially involved in hepatic thyroid hormone metabolism. In some instances, the pattern of regulation was similar to that seen in rodents, for example up-regulation of Cyp3A4, SULT1B1, SULT1A1, SYLT1C2. In other instances, the pattern differed between rodent and human. For example, deiodinase I gene expression was shown to be upregulated in response to fipronil in human hepatocytes, while it was not significantly affected in the mouse liver (Roques et al., 2013).

Although this study was limited to gene expression *in vitro* and did not address protein expression and/or functional endpoints, those data indicate that human hepatocytes cells might, as rodents, respond to fipronil exposure through hepatic enzyme induction including pathways that are related to TH metabolism. However, nothing in the study allows the reader to determine if the pattern of exposure of the primary hepatocytes is similar to hepatic exposure *in vivo*, in particular in terms of fipronil-sulfone. In addition, if it is clear that there are qualitative similarities between human and rodent in the way thyroid-related hepatic metabolic pathways respond to fipronil and/or its metabolite exposure, there is so far no available data allowing a quantitative assessment of the similarities/discrepancies between the two species. All in all, the changes found in transcript levels in response to treatments will require further research to understand their importance in overall cellular, organ, and organismic function (Mitchell et al 2016).

5.1.5.4 Is thyroid disruption in rats, mediated through increased liver catabolism, of toxicological relevance for humans?

When performing toxicity studies in the rat, the tacit assumption is that the results **can** be used for human risk assessment, unless the adverse effects are caused by mechanisms which have specifically been shown not to be relevant for humans. This

[1] RIC_{20}: the concentration of the test chemical showing 20% of the antagonistic activity of 1 x 10^{-8} T3 via TR_{β}

may possibly be applicable to the case of thyroid tumors in rats, in relation to identifying human carcinogens. In rats, a long period of overstimulation of the thyroid gland by increased TSH due to TH insufficiency will lead to thyroid tumor formation. So far, thyroid tumor development due to overstimulation of the thyroid gland by TSH has been considered unlikely in humans, however given both the epidemiological evidence and the considerations discussed in the case study dealing with thyroid tumors, this opinion may need to be revised.

But apart from the discussions of thyroid carcinogenesis, possible species differences in relation to thyroid tumors are often used to dismiss all thyroid related effects in rats as being irrelevant for humans. An example of this was found in the Fipronil DAR, where the following text is included in the discussion of the repeated dose/oncogenicity study in rats (DAR 2004 -Annex B6, p. 155): *"The thyroid gland in rats is particularly sensitive to disturbances in thyroid hormones compared to the human thyroid. Therefore the effects in this study, particularly the increase incidence of follicular cell tumors at the highest concentration, may have no relevance to humans".* This and similar arguments are often put forward in chemical registration dossiers and pesticide DARs, in order to dismiss not only thyroid cancer findings in rat studies, but all other indications of thyroid disruption.

The toxicological stance of dismissing the rat as a predictor for any thyroid effects in humans is at least partly based on the premise that rats are believed not to have thyroxin binding globulin (TBG). However, TBG is expressed in rats, but varies according to age. In adult rats, its levels are low between 2-7 months of age (Savu et al 1991, Vranckx et al 1990, & 1989, Savu et al 1987) and transthyrethin (TTR) is the dominant T4 plasma carrier in adult rats. TTR has a lower affinity for T4 and is therefore less efficient in binding T4 than TBG. The primary function of carrier proteins is extrathyroidal storage of thyroid hormones to ensure sustained hormone action across tissues, and this contributes to the control of TH homeostasis and protection from peripheral elimination (Hurley 1998). Thus, in species in which TBG is not the major T4 carrier, the free fraction of the T4 is larger than in humans and total T4 half-life is shorter (around 24h in rats, versus 5-6 days in humans) (Lewandowski et al 2004). For these reasons, the toxicological effects on thyroid function that derive from increased thyroid hormone catabolism in the rat have often been considered irrelevant to humans. However, even though TBG is the major and specific transport protein of thyroid hormones (TH) in humans, it has recently been shown not to be the physiologically most relevant (Alshehri et al. 2015). The most physiologically relevant carrier protein in humans is also TTR. Furthermore, the differences in half- lives of the active hormone T3 between rats and humans are much smaller than for T4, as T3 half-lives for humans are 22-24 h (Jonklaas et al 2015) and around 6h in rats (Lewandowski et al 2004). Based on these more recent findings, the previous assumptions about large qualitative species differences in thyroid hormone economy between rats and humans may be incorrect, and should not be used to dismiss the rat as a predictor for all thyroid related effects in humans.

Furthermore, for TH insufficiency observed in the rat (as a result of induction of hepatic metabolism) to be dismissed as irrelevant to human risk assessment, robust results showing that liver enzyme induction leading to increased thyroid hormone (TH) clearance cannot occur in humans, would be needed. Such observations however do not exist. On the contrary, there is some evidence that chemical agents (primarily drugs) capable of inducing hepatic metabolism can affect T4 levels in humans. In a review paper by Ennulat et al (2010) it is stated that: *"Effects of hepatic drug metabolizing enzymes (DME)-inducing agents on circulating thyroid hormone levels have been reported in a few human studies. In particular, the UGT1A enzymes facilitate T4 excretion and are induced by a wide variety of agents that activate human PXR, CAR, and/or AhR (Xie et al. 2003; Kato et al. 2008). Accordingly, decreased serum T4 and free T4 (fT4) in volunteers given rifampicin alone or with Phenobarbital or antipyrine for fourteen days were attributed to increased UGT-mediated T4 turnover (Ohnhaus and Studer 1983)"*. Ennulat et al. conclude that *"(…) the collective human literature suggests that little to no effect on thyroid function occurs in otherwise healthy subjects given DME-inducing agents. However, in persons with underlying thyroid conditions, increased incidence of*

hypothyroidism has been infrequently reported in association with phase II enzyme-inducing therapies (Takasu et al. 2006)". The literature also provides evidence that this MIE can occur in humans, from studies of antiepileptic drugs. For example, the effect of the drug 5,5'-diphenylhydantoin was examined in healthy volunteers, and it was shown that during oral administration, plasma thyroxine concentration decreased to about 80% of its pretreatment level, due to increased hepatic metabolism (Larsen et al 1970).

Together, these studies indicate that we should not dismiss this mode of action as irrelevant for humans.

5.1.5.5 Should adverse effects on the HPT-axis be viewed as endocrine disruption, if caused by increased liver catabolism?

In relation to the importance of the mode of action by which a chemical induces thyroid disruption, a recent Joint Research Centre publication (JRC 2016) stated that:

"*Histopathological findings in rat thyroid and increased thyroid weight in presence of liver histopathology (including liver enzyme induction) were attributed to a liver-mediated mechanism not considered to be ED-mediated. Since in the frame of this screening methodology enhancement of the metabolism and excretion of thyroid hormones by the liver was not considered as an endocrine MoA, such effects were not considered relevant to conclude on ED"*

This statement is probably included in the JRC report because increased TH clearance due to liver effects is often considered as secondary, and not a primary endocrine disrupting effect. Hence, it would be possible to protect against TH insufficiency by regulating in relation to a "liver threshold" below which hepatic enzymes are not induced and consequently TH insufficiency cannot occur. On the other hand, an endocrinological perspective would not class TH clearance as a "secondary" effect, and rather evaluate this in the context of a balance between hormone-synthesising and –removing processes. From this point of view, it would be unusual and problematic to discard hepatic effects leading to increased TH clearance as "secondary" or not relevant for designating a chemical as a thyroid disruptor. Additionally, activation of liver enzymes by a chemical could be reversible and/or not viewed as adverse; however, effects on target tissues (e.g. foetal brain) of thyroid hormone insufficiency during development caused by activation of liver enzymes in a pregnant animal would not be reversible and would not be considered "non-adverse" (Bellanger et al 2015).

5.1.5.6 How to interpret significant reductions in circulating T4 level in rats, in the absence of other effects on thyroid endpoints?

Another relevant example from the Fipronil DAR could be used for the discussion of the relevance of T4 decreases without any other concurrent effects on the thyroid hormone system. In the discussion of the repeated dose/oncogenicity study in rats (DAR 2004 - Annex B6, p. 155) it is concluded that *"slight [though significant and dose-related] effects on circulating T4 were noted at 0.5 and 1.5 ppm [the lowest tested doses] but in the absence of any morphological alterations and any concurrent effects on T3 or TSH, are not considered to be of toxicological importance*". This argument is also often seen in other chemical registration dossiers or pesticide DARs, in relation to interpretation of possible thyroid hormone disturbances.

One example of a compound which in rat studies has been shown to effectively reduce T4 levels, but only seems to induce very few other effects on the HPT-axis is the biocide triclosan. Triclosan has some years ago been shown to up-regulate both mRNA expression and activity of some phase I and phase II hepatic (PROD and UGT) enzymes in *in vivo* studies (Paul et al., 2010a), but more recent studies have shown that triclosan also has a very high affinity for TRR, actually higher than T4 itself (Weiss et al 2015). The key events leading to reduced T4 levels after triclosan exposure could therefore be displacement of T4 from TTR, together with induction of liver enzymes that induce increased T4 clearance and lower circulating T4 levels.

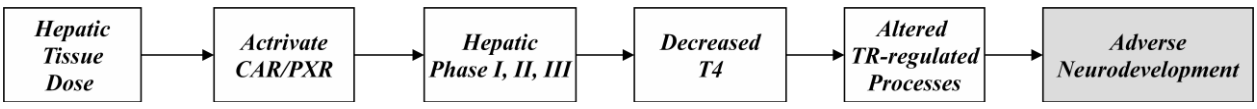
Several *in vivo* triclosan studies have been performed at the US EPA, using different strains of rats and exposure periods. These studies have revealed that even a few days of triclosan exposure reduces circulating T4 levels in rats. Doses of and above 100 mg/kg bw/day caused marked T4 decreases in the first studies (Crofton et al. 2007, Paul et al., 2010a), whereas results from a uterotrophic assay showed that even doses of 18.75 mg/kg bw/day and above for three days, significantly reduced the levels of both free and total T4 (Stoker et al 2010). In studies investigating prolonged (3-5 weeks) exposure to triclosan, doses of 30 mg/kg bw/day and above were shown to cause marked reductions in circulating T4 levels (Zorilla et al, 2009, Stoker et al. 2010).

In a developmental toxicity study, Paul et al (2012) found significant decreases in T4 levels in dams on PND22 which had received triclosan at doses of 100 mg/kg bw/day and above during gestation and lactation. In the offspring, serum total T4 levels were significantly decreased in GD2O fetuses from the 100 and 300 mg/kg bw/day treatment groups, and in PND4 pups from the 300 mg/kg/day treatment group, but there were no effects on serum T4 levels in PND14 or PND21 offspring.

This unique pattern of hypothyroxinemia, where serum T4 levels were decreased in fetuses and neonatal pups but not in older offspring had previously been seen in another developmental toxicity study of triclosan (Paul et al 2010b). This indicates that toxico-kinetic/dynamic factors could have either reduced exposure or reduced the toxicological response in the offspring during the lactation period (Paul et al 2010b). Since serum and liver concentrations of triclosan demonstrated greater fetal than postnatal internal exposure, consistent with the lack of T4 changes in PND14 and PND21 offspring, this indicated that triclosan was probably only minimally transferred to the offspring through maternal milk (Paul et al 2012), but indeed argues for greater caution in terms of foetal exposure during pregnancy. A study by Axelstad et al (2013) confirmed this suspected minimal lactational transfer, showing that while triclosan exposed dams (50 and 150 mg/kg/day) had low T4 levels during gestation and lactation, significant T4 decreases were not seen in the offspring on PND 16 in this study, but could be obtained if triclosan exposure was performed directly to the pups in the postnatal period (direct dosing from PND 3-16).

In several of the above-mentioned studies, measurements of T3 levels were also performed, but in most of the studies were not found to be significantly affected by triclosan exposure, and the same holds true for TSH levels. T3 levels were however decreased at 300 and 1000 mg/kg bw/day in a study by Paul et al (2010). TSH levels were in this study not affected at doses up to 1000 mg/kg bw/day. The only other thyroid effect observed in the in vivo studies, was altered thyroid histopathology in one study, at a 10 times higher dose than the dose causing significant reductions in T4 (i.e. 300 mg/kg bw/day for 5 weeks) in the study by Zorilla et al (2009). Triclosan therefore primarily seems to affect T4 levels and not the entire HPT-axis.

Thus, the question in relation to chemical regulation is how to deal with such compounds. Based on a FIFRA Scientific Advisory Panel meeting in 2011 a report has been published by the US EPA (US EPA 2011. From the (at that time) available experimental data on triclosan, with decreases in T4 as the critical effect in rat studies, an adverse outcome pathway for the effects of triclosan has been proposed, as shown in the figure below (US EPA 2011).



5.1.5.7 Proposed adverse outcome pathway (AOP) for triclosan effects on the thyroid hormone system

According to this view, and based on human evidence linking developmental hypothyroxinemia with altered brain development in children, increased catabolism of thyroid hormones in rats (manifested as decrease in total levels of T4) is in this AOP linked to adverse neurological development (US EPA, 2011). Because the fetus is dependent on maternal supply of thyroid hormones, decreased maternal T4 in early pregnancy means less T4 available for transfer to foetus. This could lead to decreased T4 transfer to foetal brain, which could consequently have adverse neurodevelopmental effects.

In the US EPA report (2011) the following argumentation for this causal link is used: *There are multiple data sets associating the degree of disruption of thyroid function to adverse neurodevelopmental outcomes where the perturbations are mild to moderate. Haddow et al (1999) reported a 25% decrease in maternal free T4 during the second trimester in women and associated this with neurodevelopmental and cognitive eficits in children. Henrichs et al. (2010) associated maternal hypothyroxinemia with higher risk of verbal and nonverbal cognitive delay in early childhood. It should be noted that a 10% change is within normal experiment-to-experiment variation in control values, whereas the benchmark dose response (BMR) of 20% provides a more reliable BMR. The range of 10-20% is interpreted as a balance between the biological and statistical significance of the reported changes in T4 and associated adverse outcomes.*

This reasoning is in contrast to the argument from the Fipronil DAR (but often encountered in other contexts as well) that *Decreases in circulating T4 in the absence of any morphological alterations and any concurrent effects on T3 or TSH, are not considered to be of toxicological importance.*

Whether the experimental evidence for the link between decreased T4 and adverse effects on neurodevelopment is strong enough, for the authorities to regulate chemicals solely on the basis of decreased circulating T4 levels in rats, is therefore also a very important aspect to discuss in the present case study.

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5.2 MANCOZEB CASE STUDY

5.2.1 Summary

The mancozeb case study can be used as a starting point for exploring and discussing the consequences of exposure to thyroid disrupting chemicals on the developing nervous system, i.e. explore the consequences of maternal (and early postnatal) thyroid hormone insufficiency in rats.

Mancozeb is a fungicide from the dithiocarbamate family. These pesticides are widely used for the protection of fruits, vegetables, and field crops from fungal diseases. The main degradation product of many of the dithiocarbamates is ethylene thiourea (ETU) (WHO 1988), a compound that exerts various toxic effects in rats. The mode of action is believed to be through inhibition of the enzyme thyroid peroxidase (TPO) (Marinovich et al. 1997). The diminished production of T3 and T4 consequently increases TSH (thyroid stimulating hormone) release. The action of TSH causes thyroid follicular cell hypertrophy, changes in cell shape and loss of colloid from the thyroid follicle and increased thyroid weight, and conditions of prolonged exposure in rats leads to tumours of the thyroid gland.

Mancozeb is a commonly sold fungicide in many countries, including Norway (Nordby *et al.*, 2005), and the United States (Acquavella *et al.*, 2003). In an American agricultural health study, mancozeb exposure was strongly associated with increased incidence of thyroid disease in female spouses of pesticide applicators (Goldner *et al.*, 2010), but most human studies have not shown any association between mancozeb exposure and adverse health outcomes.

A vast number of repeated-dose toxicity and carcinogenicity studies have been performed with mancozeb, most of them in rats, but also in mice and dogs. Most of these studies have not been published in the open literature, and therefore assessment of this data was performed based on the summaries available in an EU draft renewal assessment report (DRAR, see Annex 1 & 3). The repeated dose toxicity studies have collectively shown that the thyroid is the major target organ of mancozeb, and that mancozeb, through its degradation to ETU, exerts numerous adverse effects related to the function of the thyroid gland.

The main aim of the present case study is to discuss the consequences of developmental exposure to a clearly thyrotoxic compound like mancozeb on neural development in the offspring, i.e. explore the consequences of maternal TH insufficiency in rats. This scientific problem has been investigated thoroughly in toxicity studies where developmental hypothyroidism has been caused by maternal PTU exposure or by thyroidectomy, and here the consequences for the offspring are quite clear (delayed development, decreased postnatal body weighs, hearing impairment, altered motor activity levels and deficits in learning and memory)(Akaike et al 1991, Brosvic et al 2002, Noda et al 2005, Goldey et al 1995, Kobayashi et al 2005).

However, in contrast to what would be expected based on the clear disruptions by mancozeb of the thyroid hormone axis, no adverse effects on nervous system development have been observed in two developmental mancozeb studies (Beck 2008 in DRAR, see Annex 1, Axelstad et al 2011). Additionally, an extended one generation reproductive toxicity study (EOGRTS) with a DNT cohort has also been conducted with ETU, and here no adverse effects on brain development were seen either (Marty et al 2013b, in DRAR).

Therefore, the questions discussed in the present case study include the following:
Why do marked maternal T4 reductions produced by certain chemicals lead to severe developmental neurotoxicity in the offspring, but not when such changes are produced by other chemicals, even though maternal T4 decreases during gestation are comparable? When are the critical windows of brain development in rats, i.e. does hypothyroxinemia have to occur both pre- and postnatally, if behavioral effects are to be evident in the rat offspring? And are the present OECD test guidelines adequate for assessing the possible adverse effect on brain development caused by thyroid disruption, and if not, how could they be updated to include more sensitive downstream bioassay endpoints?

5.2.2 Scope and aims of this case study

In two developmental toxicity studies in rats, maternal TH insufficiency induced by macozeb exposure during gestation and lactation did not lead to any adverse neuropathological effects, or observable behavioral abnormalities in the offspring. Thus, the mancozeb case study offers the opportunity of discussing the consequences of maternal TH insufficiency for the offspring, as well as the test methods presently used to assess this issue. The aspect of thyroid disruption which will be discussed in this case study are the downstream effects of maternal hytothyroxinemia/hyperthyroidism caused by a TPO inhibitor. More specifically, the following questions will be addressed, both in relation to their scientific implications and their regulatory significance/impact:

- Why do marked maternal T4 reductions (e.g. ~50%) lead to severe developmental neurotoxicity effects when these are caused by PTU exposure, but not when caused by mancozeb (or ETU)? Since mancozeb exerts its thyroid disrupting effects through the same mode of action as PTU (inhibition of TPO), some aspects other than just the mode of action by which thyroid disruption is obtained, must be contributing to this difference in outcome.
- When are the critical windows of brain development in rats, and is insufficient milk transfer of certain compounds the cause of these differences – i.e. does marked hypothyroxinemia/ hypothyroidism have to occur in both the pre- and postnatal period of rat brain development, if behavioural effects are to be evident in the rat offspring?
- Or is brain development in the rat offspring in reality affected by all compounds causing prenatal hypothyroxinemia, but can the more subtle effects not be seen when using the test methods that are presently recommended/mandatory in OECD neurotoxicity guidelines?
- What could be gained by updating the DNT guideline (along with the DNT cohort in the EOGRTs) with assessment of more sensitive downstream bioassay endpoints, and what should those be?

In order to address these discussion points, the present case study includes information from mancozeb studies found in the open literature, and much data from the DRAR for Mancozeb with studies not available in the open literature. It is important to bear in mind that the presented case is not meant to be an exhaustive substance review, so only data relevant to the above mentioned specific discussion points has been presented.

5.2.3 Mancozeb and its uses

Mancozeb is a fungicide from the dithiocarbamate family. These pesticides are used for protection of fruits, vegetables, and field crops from fungal diseases worldwide (WHO, 1988). Mancozeb is a very commonly sold fungicide in Denmark (Danish EPA, 2010), Norway (Nordby *et al.*, 2005), and the United States (Acquavella *et al.*, 2003), and a recent publication shows that there are subpopulations around the worldwho experience very high mancozeb exposures (Van Wendel de Joode et al 2014). In this study 445 pregnant women from Costa Rica, living in areas where mancozeb is aerially sprayed over banana plantations, were shown to have more than five times higher ETU concentrations in their urine, than those reported for other general populations. They also found that seventy-two percent of these women had estimated daily intakes above the RfD.

5.2.4 Observations from human studies with relevance to thyroid disruption

In 1997 Steenland et al published a paper investigating the effects of dithiocarbamate exposure on human thyroid hormone levels. Their relatively small study included 49 heavily exposed Mexican workers who were spraying with mancozeb without protective equipment, 14 lightly exposed landowners and 31 non-exposed controls. Urinary ETU levels were used to compare exposure between groups. They found an increase in TSH ($p = 0.05$) among applicators compared to controls, which could indicate that effects on the thyroid gland of heavily exposed workers may occur, however no decreases in thyroid hormone (T4) were seen. In the American Agricultural Health Study, the authors examined the cross-sectional association between the use of several different pesticides and the risk of thyroid disease among female spouses (n = 16,529), and found that use of the fungicide maneb/mancozeb, was significantly associated with hypothyroidism (OR(adj) = 2.2 (95% CI: 1.5, 3.3) (Goldner et al 2010). A large Norwegian study based on the National registers in Norway (105,403 female and 131,243 male farmers, born in 1925-1971, and their 300,805 children, born in 1952-1991) has shown a moderate association between mancozeb exposure and neural tube defects in newborns from farmer families, but no association between mancozeb exposure and thyroid cancer (Nordby et al., 2005). A few other epidemiology studies have also investigated associations between dithiocarbamate exposure and thyroid cancer, but have found none (De Fonso; 1976; Charkes et al, 1985; Vogel, 1990 in DRAR)

In a recently published Italian epidemiology study (Medda et al 2017) the aims were to i) to evaluate thyroid effects of exposure to mancozeb in a sample of Italian grapevine workers, and ii) to verify whether the iodine intake may modulate the risk of thyroid disruption due to the mancozeb metabolite ethylenthiourea (ETU). 177 occupationally exposed male workers (29 from Chianti, a mild iodine deficient area, and 148 from Bolzano an iodine sufficient province) and 74 non-occupationally exposed male controls (34 from Chianti and 40 from Bolzano) were enrolled in the study. Serum biomarkers of thyroid function, as well as urinary iodine and ETU concentrations were assessed. Moreover all the recruited subjects underwent clinical examination and thyroid ultrasound. Multivariate comparisons showed lower mean serum levels of FT4 in Chianti-workers as compared to Bolzano-workers. Moreover, an increased urinary iodine excretion (>250µg/L) was more frequently found among more exposed workers (ETU>20µg/L) than among less exposed ones and this effect was more pronounced in Chianti- than in Bolzano-workers. Chianti-workers also showed a significantly higher frequency of very low thyroid volume (≤6.0ml) as compared to controls. These findings indicated a possible mild thyroid disrupting effect due to occupational exposure to mancozeb, which was more pronounced in workers residing in an area characterized by a

mild to moderate iodine deficiency as compared to workers residing in an area covered by a long-lasting iodine prophylaxis program.

5.2.5 Observations from experimental studies

5.2.5.1 In vitro and mechanistic studies investigating thyroid disrupting mode of action

The *in vitro* accivity of ethylenethiourea (ETU), the main metabolite of mancozeb, was tested in chinese hamster ovary (CHO) cells transfected with human TPO gene, at concentrations of 0.1 to 50 µM. *In vitro* cytotoxicity was assessed together with the effect on the thyroid peroxydase (TPO) activity. Shortly after ETU exposure to concentrations of 5 and 50 µM, TPO iodinating activity was markedly reduced, while cytotoxicity was unaffected, indicating that ETU blocks TPO iodinating activity even at low concentrations (Marinovich et al, 1997).

Generally, in all of the performed *in vivo* studies with mancozeb and ETU, effects on circulating T4 levels tend to be seen at lower doses than those showing effects on thyroid weight and histopathology. This is consistent with TPO inhibition (and consequent reduction of T4) being the molecular initiating event (MIE).

5.2.5.2 ADME studies on mancozeb and investigations of thyroid disrupting effects of ETU

From the different ADME studies described in the DRAR, it appears that in mammals, mancozeb can be absorbed orally, dermally and by inhalation. It is rapidly metabolised into its breakdown products, the main one of these being ethylene thiurea (ETU) as demonstrated in rat, cow and human. ETU is notably found in several organs, but with the thyroid gland showing the highest level of ^{14}C-containing residue. ADME studies have shown that the breakdown of mancozeb in rats and cows is quite similar. In cows, the level of ^{14}C- containing residue in milk was about 1.5% of the ingested concentrations. Whether a similar percentage is transferred to the maternal milk of in rats and humans has not been investigated.

Several studies in the DRAR describe how ETU is toxic to the thyroid hormone system in rats and other test species. Below is a short summary of these test results. *In the rat, ETU has been shown to affect the thyroid hormone system in the majority of the available repeated-dose toxicity studies. After ETU administration for 28 or 90 days (6 studies in total), significant T4 and T3 decreases were seen at doses from 6-15 mg/kg bw/day and higher, whereas dosing for up to 120 days resulted in an effect level of 5 mg/kg bw/day. All studies showed significant T4 decreases as the 'first' effect and altered thyroid weight, histopathology and TSH levels at the same or somewhat higher doses. In studies where ETU was administered for 2 years, changes in T4 and TSH were seen at doses from 1.25 mg/kg bw/day. Further evidence of disturbance of the HTP axis occurred at higher doses, with histopathological changes and thyroid follicular adenomas and carcinomas appearing at doses from 38 mg/kg bw/day in one study and from 9 mg/kg bw/day in another.*

In the mouse thyroid, ETU caused a similar spectrum of changes to those seen in the rat. In 28- and 90-day studies, changes in thyroid hormone levels, thyroid weight and histopathological findings were seen at doses from 17-20 mg/kg bw/day, and in a 2-year carcinogenicity study, ETU caused increased incidences of thyroid follicular adenomas and carcinomas at doses from 50 mg/kg bw/day. Administration of ETU to dogs and monkeys also caused disturbance of the HTP axis. After 52 weeks of ETU dosing in dogs, thyroid effects were seen at doses of 2 mg/kg bw/day and above, whereas ETU administration to monkeys caused adverse effects on T4, T3, TSH, thyroid weight and histopathology at dose of 2.5- 7.5 mg/kg bw/day.

Based on this review of ETU data, there is no doubt that ETU, the main metabolite of mancozeb, disrupts the thyroid hormone system. The fact that adverse effects on the thyroid are seen in all the tested species (rat, mouse, dog and monkey) indicates that the mechanism of TPO inhibition is probably also a relevant MIE in humans.

5.2.5.3 Repeated dose toxicity studies investigating thyroid effects of mancozeb in rats

The effects of Mancozeb seem very similar to those seen after ETU exposure, however, the doses needed to obtain these effects seem to be somewhat higher than for ETU. Flippin et al. (2009) dosed weanling female rats (8-16 per group) with 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 or 1000 mg mancozeb/kg bw for 4 days, and 24 hours after the last dose, serum T_4 and liver microsomal enzyme activity was assayed. Mancozeb produced a dose-dependent decrease in circulating levels of T_4 with statistically significant decreases from 31.3 mg/kg and an ED_{50} of 259 mg/kg bw/day. No induction of liver enzyme activity of EROD (ethoxyresorufin O-deethylase), PROD (pentoxyresorufin O-deethylase) or T_4-UDPGT (uridine diphosphate glucuronyl transferase) was seen.

The majority of toxicity studies on Mancozeb are not publicly available, and therefore the following summary is based on information from the DRAR. *No 28-day studies investigating the effects of mancozeb on the thyroid hormone system in rats exist. In 90-day studies effect doses leading to T4 reductions, increases in TSH and in thyroid weight have been shown to be at 15, 28 and 57 mg/kg bw/day in three different studies. Repeated inhalation exposure of rats to mancozeb aerosol for 13 weeks, has also been shown to reduce serum T4 levels and cause thyroid hyperplasia. Furthermore, three carcinogenicity studies with mancozeb have been performed in rats. In one carcinogenicity study, treatment-related effects on the thyroid (including thyroid adenomas and carcinomas) were seen at the top dose (~30-40 mg/kg bw/day), whereas no effects were seen at the lower dose (~5-7 mg/kg bw/day). In another guideline carcinogenicity study, decreased T4 levels and altered thyroid histopathology (but no tumourigenic response) was seen at the top dose of ~17-21 mg/kg bw/day, with no effects at ~4-5 mg/kg bw/day. The lack of thyroid tumours in this study could be explained by the fact that the top dose was lower than in the first study.*

A cancer study was also conducted by Belpoggi et al. 2002. Groups of 75 male and female Sprague-Dawley rats, were administered mancozeb at the concentration of 10, 100, 500 or 1000 ppm in feed for 104 weeks. Malignant thyroid gland tumours were increased in males at 500 and 1000 ppm, and in females at 10, 100 and 1000 ppm mancozeb. The study had some shortcomings but confirmed that mancozeb can cause thyroid follicular carcinomas in rats.

Also two multi-generational studies performed in rats were described in the DRAR. In the first one, SD rats received mancozeb doses of 30, 120 or 1200 ppm for two generations. In parental animals the highest dose (corresponding to 70 mg/kg bw/day) caused decreased body weight and food consumption, increased thyroid weights and altered thyroid and pituitary histopathology. In the other study, SD rats received mancozeb doses of 25, 150 or 1100 ppm for two generations. At 1100 ppm (65 mg/kg bw/day) mean thyroid weight was markedly increased in the males of both generations and moderately increased in females of both generations. Histopathology, however, revealed thyroid hyperplasia and hypertrophy in nearly all parental animals of the F_0 and F_1 generation at the the high dose. Thyroid follicular cell adenomas were also found in five males of the F_0 generation and in 11 males of the F_1 generation at the high dose. No thyroid effects were seen at the 150 ppm dose (~7 mg/kg bw/d).

Together, these studies clearly show that in rats, mancozeb has very similar effects to those of ETU, but that effects seem to occur at marginally higher doses (i.e effects doses seem to be ~15-75 mg/kg bw/day). As with ETU, the longer the exposure time used, the lower the doses needed to induce effects.

5.2.5.4 Repeated dose toxicity studies investigating thyroid effects of mancozeb in other species

Repeated-dose toxicity studies in mice and dogs have shown that in these species the thyroid is also the major target organ of toxicity. *The DRAR describes some 28- and 90-day studies in mice, where increased thyroid weights and thyroid hyperplasia was seen at*

doses from 150, 180 and 200 mg/kg bw/day, respectively. In an 18-month carcinogenicity study, T4 was reduced at doses of 131 mg/kg bw/day, whereas effects on T3 were equivocal, TSH was unaffected and thyroid tumours were not seen.

In the dog, the thyroid hormone system is also adversely affected by mancozeb treatment. After 3-months administration, T4 and T3 levels were reduced, thyroid weight was increased and thyroid follicular cell hypertrophy was seen at doses of 30 mg/kg bw/day and above. Two longer term studies (52 weeks) were conducted in dogs and gave similar results to the 3 month study. Effects on T4, thyroid weight and histopathology were seen at doses of 23 mg/kg bw/day and above in one study and at 50 mg/kg bw/day in the other. Thyroid tumours were not seen in any of the dog studies.

Based on these data, the mouse thyroid appears to be less sensitive to mancozeb exposure than that of the rat, whereas the thyroid hormone system of dogs seems as sensitive as in rats. No thyroid tumours were seen the mouse and dog studies, but since ETU exposure has been shown to cause thyroid tumours in mice, it is possible that the tested mancozeb doses were simply not high enough to induce tumour formation in the mice.

5.2.5.5 Developmental toxicity studies investigating thyroid disruption and neurotoxicity of mancozeb and ETU

In toxicity studies where developmental hypothyroidism has been caused by maternal PTU exposure or by thyroidectomy, the consequences for the offspring are quite clear and include delayed development, decreased postnatal body weighs, hearing impairment, altered motor activity levels and deficits in learning and memory (Brosvic et al 2002, Noda et al 2005, Goldey et al 1995b, Kobayashi et al 2005). In order to assess whether the mancozeb-induced hypothyroidism would affect brain development, two developmental neurtoxicity studies have been performed with Mancozeb, and one with ETU. Unexpectedly, none of them have shown any adverse effects on neurobehavioural development, even though the tested doses resulted in thyroid hormone disruption in the dams.

A guideline developmental neurotoxicity study on mancozeb (performed according to OECD TG 426) was conducted to address the concern about the potential relationship between thyroid effects and adverse brain development (DRAR). The study consisted of a range-finding study with focus on measurements of thyroid endpoints (Beck, 2008a), and a final study assessing neurodevelopmental and neuropathological endpoints (Beck 2008b). A detailed study description is included in Annex 1, for those readers interested in assessing the study results in more detail. Unfortunately, detailed results were only provided in the DRAR for some of the endpoints (thyroid hormone levels, thyroid histopathology and body weight gain) whereas no figures or tables showing results from the behavioral or neuropathological assessment were included. As the study report was not available to us, we have to trust the conclusion from the DRAR that indeed no effects indicating developmental neurotoxicity were observed.

In the range-finding study, mancozeb was administered in the diet at target doses of 0, 5, 30 and 60 mg/kg/d to female SD rats (n=15) from GD 6 to GD 20 or PND 21. Dams given 60 mg/kg bw/day of mancozeb showed signs of general- and thyroid toxicity and the offspring in this group had lower body weights and reduced T4 levels on GD20 and PND4. The dose of 30 mg/kg caused moderately decreased maternal weight gain during gestation, higher mean serum concentrations of TSH, lower mean serum concentrations of total T_4 and a marginally higher incidence of thyroid gland follicular cell hypertrophy. In the offspring, no significant effects on body weight or thyroid endpoints were seen. There were no treatment-related effects at 5 mg/kg/day.

Based on the results of the range-finding study, dietary mancozeb doses of 5, 15 and 30 mg/kg bw/day were chosen for the DNT study. Here, dams were exposed from gestation day 6 through to weaning, and the offspring were assessed in a number of neurobehavioural test and neuropathological analyses. In the highest dose group, maternal body weight gain was reduced following the onset of treatment. For gestation

days 6-20, the decrease in body weight gain was 4.6%. However when litter size was used as a covariate in the statistical analysis of body weight gain, the reduction at 30 mg/kg bw/day was 9.4%. In the range finding study the decrease in adjusted weight gain was 11%, at the same dose level. This indicates some degree of maternal toxicity at this dose level. Furthermore, in these high dose dams absolute and relative mean thyroid weights were increased by 7.5% and 9.1%, respectively, and corresponded with an increased incidence of thyroid follicular cell hypertrophy; (6/24 dams with minimal follicular cell hypertrophy in controls vs. 11/25 in the high dose group). Whether the changes in thyroid weight and histopathology were statistically significant is not stated in the DRAR.

In the previously reviewed repeated dose toxicity studies with Mancozeb doses above 30 mg/kg were needed to induce T4 reduction after 4 days of exposure, whereas doses of 15-75 mg/kg bw/day for 90 days have been shown to cause adverse effects on the thyroid (DRAR). This indicates that the dams in the present study were exposed to mancozeb doses that were in the low range of those previously shown to affect the thyroid hormone system after such a relatively short term exposure (GD6-PND 20= 35 days). There were no test substance-related effects on any of the F1 litter parameters including survival, clinical signs, FOB, growth, development, motor activity, startle response, learning and memory, brain morphometry and histopathology of the central and peripheral nervous systems (though no results were shown in the DRAR). Based on these findings, it was concluded that mancozeb had been adequately tested for DNT and that mancozeb was not a developmental neurotoxicant. Whether this conclusion can indeed be drawn based on the available data is discussed in the next chapter, together with discussion of the results from the other developmental neurotoxicity study on mancozeb (Axelstad et al 2011), and the EOGRTS study on ETU (Marty et al 2013b in DRAR).

A study by Axelstad et al (2011) was also conducted to investigate if perinatal mancozeb exposure would cause neurobehavioural effects in rats. A detailed description of this study is provided in Annex 2. Wistar rats (n=9-21) were exposed to mancozeb at doses of 50, 100 or 150 mg/kg bw/day from gestation day 7 until lactation day 16. Because of systemic toxicity in the dams (body weight loss and hind limb paralysis) the high dose was reduced from 150 to 100 mg/kg on GD 16. Blood samples were collected for T4 analysis from dams on GD15 and PND24 and from pups on PND16 and PND24. Thyroid glands were weighed and processed for histopathological evaluation from offspring on PND 16 and from dams on PND 24. Offspring were examined for motor activity on PND14, 17, and 23 (one male and one female pup per litter was assessed). On PND 24, 1-2 animals per sex from each litter were weaned, and used for further behavioural analysis. The behavioural tests were performed in 2-7 month old offspring, and included assessment of spatial learning in a radial arm maze, motor activity measurements in activity boxes and acoustic startle response.
Despite clear maternal hypothyroxinemia (T4 decreases on GD 15 of 22, 27 and 37% in the three dose groups respectively), no significant effects were seen in offspring behaviour or acoustic startle response, in young or adults animals, and no dose-dependent trends were seen. There was no effect in dams on PND 24 thyroid weights or histopathology, but this could be because these endpoints were assessed 8 days after exposure had stopped. In offspring no treatment-related effects on T4, thyroid gland weight or histopathology were seen on PND 16. The authors hypothesized that low transfer of mancozeb to the maternal milk could explain why no thyroid effects were seen in the offspring and why no behavioural effects were observed.

A third study not showing any adverse neurobehavioural effects was performed with ETU. In an extended one generation toxicity study (performed according to OECD TG 443) ETU was administered to Sprague Dawley rats (27/sex) at dose levels of 0, ~0.2, 2.0 and 10 mg/ kg bw/day (0, 2.8, 28, 140 ppm) for approximately four weeks prior to breeding, and continuing through gestation and lactation. At weaning, F1 offspring were divided into cohorts for behavioural and neuropathological assessment. A detailed study description of this study (as shown in the DRAR) is provided in Annex 3.

ETU caused no effects on reproductive toxicity or developmental neurotoxicity up to the top dose of 10 mg/kg bw/day. Thyroid toxicity (hypertrophy in adults) was seen at the mid and high dose. No data showing any results from the many neuropathological or behavioural assessments were shown in the DRAR, but it was concluded that none of these endpoints were affected. In high dose offspring a lower brain weight was seen in Cohort 2A. This was accompanied by decreases in overall brain macroscopic and microscopic measurements. According to the DRAR conclusions growth at 140 ppm was reduced during late lactation, juvenile and early adult periods. The differences in brain weight (6-7%) were relatively small compared to the effects on body weight (up to 10%), and the morphometric analysis indicated that the brain was slightly small overall, rather than decreased in any specific region/layer. Consequently, in the absence of any effects on neurobehavioural endpoints or neuropathology, the differences in brain weight at 140 ppm ETU were considered to reflect the reduced growth during late lactation, juvenile and early adult periods and not to be evidence of specific developmental neurotoxicity.

5.2.6 Why are the expected neurobehavioural effects of hypothyroxinemia not seen in these rat studies?

Apart from the developmental studies on Mancozeb and ETU presented above, several other DNT studies investigating rodent offspring from hypothyroid/hypothyroxinemic dams have also shown that adverse behavioural outcomes are not always present. This is for instance the case with fipronil and perchlorate (both discussed in other case studies). The discussion points below will be dealing with the data from the present case study on Mancozeb/ETU, but the conclusions should be more broadly applicable.

The key issue in this context is, why do marked maternal T4 reductions (and in some cases also other adverse effects on the maternal thyroid hormone system) not lead to any behavioural effects (or other assessed neurotoxicological endpoints) in the offspring?

Listed below are some possible explanations for this question:

1) The thyroid disrupting effects of mancozeb/ETU on the dams where not severe enough to lead to altered brain development in the offspring, because the tested doses were too low.
2) The offspring were not hypothyroid themselves in the postnatal period (due to limited milk transfer) and since much brain development occurs postnatally in rats, the prenatal hypothyroxinemia was not severe enough to disrupt brain development.
3) Offspring brain development was actually affected, but the used neurobehavioural assessment methods are not suited for the detection of subtle effects in the brain, caused by maternal hypothyroxinemia (to be discussed in the next section).
4) The decreases in circulating T4 levels (due to TPO inhibition) lead to upregulation of peripheral deiodinase activity in the offspring brains, leading to a larger conversion of T4 to the active T3, which compensated for the low circulating T4 levels and provided enough T3 for normal brain development.

An equally relevant question is why there were no adverse effects on neurodevelopment seen in these three studies, when similar maternal T4 decreases caused by PTU exposure would most likely have resulted in a wide range of adverse effects. Since mancozeb/ETU exerts its thyroid disrupting effects through the same mode of action as PTU (inhibition of TPO), some aspects other than just the mode of action, must be contributing to this difference in outcome.

Some possible explanations for this problem are listed:

1) The majority of reported developmental PTU studies use very high doses of PTU and therefore cause very severe hypothyroidism. If PTU and ETU/mancozeb doses were chosen to result in an "identical" degree of hypothyroidism, an "identical" outcome regarding neurobehavioural development would also be seen.

2) PTU has other modes of action than just TPO inhibition (also inhibition of deiodinase activity). This difference is responsible for the observed differences in outcome.

5.2.7 How can OECD guidelines investigating DNT be improved to include more sensitive endpoints for adverse outcomes of thyroid disruption?

In the case study on perchlorate, data from studies investigating neurological effects of perchlorate exposure during development are shown. A more detailed description of one of these studies (Gilbert and Sui, 2008) is provided below, in order to help discussions of which downstream endpoints could be useful to include in the OECD guidelines dealing with developmental neurotoxicity.

Pregnant LE rats (106 dams used, n=26?) were exposed to 0, 30, 300, or 1,000 ppm (~ 5, 44 and 140 mg/kg bw/day) of ammonium perchlorate in drinking water from gestational day 6 until weaning. Adult male offspring were evaluated in a series of behavioral tasks and neurophysiologic measures of synaptic function in the hippocampus. At the highest perchlorate dose T_3 and T_4 levels were reduced in pups on postnatal day 21. T_4 in dams was reduced relative to controls by 16%, 28%, and 60% in the 30-, 300-, and 1,000-ppm dose groups, respectively. TSH was significantly increased in the high-dose group, whereas no changes were seen in serum T_3. No group differences were seen in any of the behavioral studies (i.e. no effect on horizontal or vertical motor activity (n=8-11), no effect on latency to find the hidden platform in the Morris water maze (n=11-17), and no differences in trace fear conditioning or conditioning to context (original test box). Brain and hippocampal weights in the offspring were measured on PNDs 4, 14, and 21 in male and female offspring, and here also no significant effects were seen. However, in the adult offspring, synaptic transmission in the hippocampus was affected by perinatal perchlorate exposure, as significant reductions in excitatory and inhibitory synaptic transmission were observed in the dentate gyrus. The dose-dependent deficits in hippocampal synaptic function were indicative of an irreversible impairment in synaptic transmission in response to developmental exposure to perchlorate (Gilbert and Sui 2008).

Additional methods for assessing neurotoxicity to those presently included in the OECD neurotoxicity guidelines, have also been used by Harry et al (2014), in an investigation of the neurotoxicological effects of perinatal exposure to the dioxin-like compound TCAB (3,3'4,4'-tetrachloroazobenzene). This chemical induced clear hypothyroxinemia, and the authors investigated guideline-like behavioural endpoints and conventional neuropathology in the offspring, but also investigate specific regions of the hippocampus, and glial cells – which have previously been shown to be very sensitive to thyroid disruption**.** The study and its conclusions can be summarized in the follwing way:

SD rats were orally treated with 0.1, 1.0, or 10 mg TCAB/kg bw/day, for two weeks prior to cohabitation until post-partum day 3, and male offspring from post-natal day (PND)4-21. In the dams, TCAB, induced dose-related reductions in serum T4 levels with no change in T3 or TSH. At PND21, the high dose animals showed a deficit in body weight gain. Conventional neuropathology detected no neuronal death, myelin disruption, or gliosis, and no effects were observed on post-weaning behavioral assessments in control, 0.1 and 1.0mg/kg/day dose groups [the 10 mg/kg group was not assessed]. However, astrocytes displayed thinner and less complex processes at 1.0 and 10 mg/kg/day. At 10 mg/kg/day, microglia showed less complex processes, unbiased stereology detected fewer hippocampal CA1 pyramidal neurons and dentate granule neurons (GC) and Golgi staining of the cerebellum showed diminished Purkinje cell dendritic arbor. At PND150, normal maturation of GC number and Purkinje cell branching area was not observed in the 1.0 mg/kg/day dose group with a diminished number and branching suggestive of effects initiated during developmental exposure. Based on this the authors concluded that the demonstrated sensitivity of hippocampal neurons and glial cells raises support

for considering additional anatomical features of brain development in future DNT evaluations (Harry et al 2014).

Taken together, the results presented in the present case study indicate that more sensitive neurotoxicity endpoints would be useful to include in the presently conducted OECD guideline studies.

If/when this is implemented, it may be possible in the future to assess how neurodevelopmental outcomes differ for different compounds, depending on their mode of action in causing thyroid disruption.

5.2.8 References

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5.2.9 Annex 1. Description of OECT TG 426 DNT study on mancozeb from the DRAR

Range-finding study (Beck 2008a)

Methods: Mancozeb was given on a continuous basis in the diet to groups of 15 bred female SD rats from gestation day 6 until euthanasia (post partum day 21). The target test substance doses of 5, 30 and 60 mg/kg bw/d and the mean calculated doses during gestation/lactation were 5/6, 29/35, 55/73 mg/kg/d. One group of animals received the basal diet alone and served as the control group. Maternal clinical observations, body weight and food consumption were monitored. Five females/group were euthanized on GD 20 for maternal and foetal blood collection for determination of mancozeb and ETU concentrations in plasma. Maternal TSH and T_4 were measured and the thyroid glands were weighed and examined microscopically. The remaining females were allowed to litter and to rear their offspring to day 21 post partum. Maternal blood samples were collected on LD4 from 5 females/group for determination of plasma mancozeb and ETU. Milk samples from 5 females per group were collected on LD4 and LD10 for determination of mancozeb and ETU. At termination on LD 21, maternal blood was collected for thyroid hormone assessment and the thyroid glands were weighed and examined microscopically. The offspring were weighed and observed at intervals. On PND 4, litters were culled to 8 pups/litter; blood samples were collected for toxicokinetic evaluation from the culled offspring in litters whose dams were used for evaluation of

plasma mancozeb and ETU concentrations. All surviving pups were euthanized and necropsied on LD 21.

Results: No test substance-related clinical findings were observed in parent females. At 60 mg/kg/d, body weight, body weight gain and food consumption were significantly decreased during the gestation treatment period (by 10.5%, 37% and 16%, respectively; $p<0.01$). Significant reductions in gestation body weight gain and food consumption were also evident at 30 mg/kg/d (by 14% and 12%, respectively; $p<0.01$). Subsequently, mean body weights in the 60 mg/kg/d group females remained lower during lactation, but there were no test substance-related effects on mean body weight gain or food consumption. No test substance-related effects on mean body weights, body weight gains or food consumption were noted during gestation in the 5 mg/kg/d group or during lactation in the 5 and 30 mg/kg/d groups. An increased incidence of thyroid gland follicular cell hypertrophy was noted in the 30 and 60 mg/kg/d groups on LD 21 and was associated with a statistically significant reduction in serum T4; increased TSH at 60 mg/kg/d did not achieve statistical significance. Relative thyroid weights were increased in some of the 60 mg/kg/d group dams and associated with follicular cell hypertrophy.

Summary of thyroid effects

	Dose level of Mancozeb (mg/kg bw/day)			
	0	5	30	60
Total T_4 (µG/dL) GD 20	1.32	0.82	0.59*	1.02
TSH (ng/mL) GD 20	9.58	12.46	11.20	11.98
Total T_4 (µG/dL) LD 21	3.94	4.36	2.98*	2.22**
TSH (ng/mL) LD 21	10.82	10.19	10.75	14.91
Absolute thyroid weight (g) GD 20	0.0233	0.0213	0.0254	0.0260
Absolute thyroid weight (g) LD 21	0.0240	0.0223	0.0216	0.0232
Thyroid cyst, ultimobranchial, present GD 20	2/5	1/5	0/5	1/5
Thyroid hypertrophy, follicular cell - minimal GD 20	0/5	0/5	1/5	1/5
Thyroid cyst, ultimobranchial, present LD 21	5/10	2/10	5/9	2/10
Thyroid hypertrophy, follicular cell - minimal LD 21	2/10	0/10	4/9	5/10

Statistically significant difference from control *P<0.05, Statistically significant difference from control **P<0.01

Mean gestation length and parturition were unaffected by mancozeb. The mean number of pups born, the percentage of males at birth, live litter size on PND 0, postnatal survival and general physical condition were unaffected by maternal exposure to mancozeb. Lower mean body weight gains were observed in the male and female pups in the 30 and 60 mg/kg/d groups during PND 4-7, 7-11 and 17-21, resulting in mean body weights that were 10.9% to 21.6% lower than the control group values during PND 7-21. These body weight decreases were most marked on PND 7 and 11, but were not dose-related.

Summary of pup body weights (g) selected days

Post natal day	Dose level of Mancozeb (mg/kg bw/day)							
	Males				Females			
	0	5	30	60	0	5	30	60
1	7.2	7.0	7.0	6.8	6.8	6.6	6.7	6.6
4 (pre-cull)	9.5	9.3	8.8	9.0	8.9	8.7	8.3	8.6
7	14.8	13.4	11.6*	12.3*	13.7	12.5	11.1	11.5
14	28.5	26.6	24.2	24.7	26.8	25.1	23.4	23.5
21	46.1	43.1	40.0	40.5	44.1	41.0	38.7	38.6

Statistically significant difference from control *P<0.05

There were no macroscopic findings in the pups that were found dead, euthanized in extremis or at the scheduled necropsy that could be attributed to maternal exposure to mancozeb. Plasma and milk analyses in the high dose animals (60 mg/kg/d) showed that the pups were exposed to residues of both mancozeb and ETU. The mean concentrations in plasma for GD 20 dams and foetuses were 0.356 ppm and 0.278 ppm, respectively. For LD 4 dam and PND 4 pup plasma, the mean concentrations were 1.18 ppm and 0.037 ppm, respectively. The mean concentration in milk was 0.426 ppm in the LD 4 samples and 0.440 ppm in the LD 10. ETU residues were found in plasma and milk from all dose groups. The ETU residue levels increased with increasing dietary concentration of mancozeb.

Conclusions: In this DNT range-finding study, mancozeb was administered in the diet at target doses of 0, 5, 30 and 60 mg/kg/d to female SD rats from GD 6 to GD 20 or LD 21. Dams given 30 or 60 mg/kg/d mancozeb had lower mean body weights, body weight gains and food consumption, higher mean serum concentrations of TSH, lower mean serum concentrations of total T_4 and a higher incidence of thyroid gland follicular cell hypertrophy which generally corresponded with increased relative thyroid weight at the highest dose. The offspring in the 30 and 60 mg/kg/d groups had decreased body weights and body weight gains. There were no treatment-related effects at 5 mg/kg/d. Therefore, dietary doses of 5, 15 and 30 mg/kg/d were selected for a definitive DNT study of mancozeb.

Full DNT study (Beck 2008b)

Method :Mancozeb was given on a continuous basis in the diet to groups of 25 bred female SD rats from gestation day 6 until euthanasia (post partum days 21-28). The target test substance doses of 5, 15 and 30 mg/kg bw/d were achieved. One group of animals received the basal diet alone and served as the control group. Clinical observations including FOB, body weight and food consumption were monitored for the females during the study. Dams were allowed to litter and rear their offspring to day 21 post partum. At necropsy, thyroid weights were collected and the thyroids processed for histopathological examination. Pre-weaning developmental landmarks (pinna detachment, eye opening and surface righting response) were evaluated in offspring. On postnatal day (PND) 4, litters were culled to 8 pups/litter (4 pups/sex, when possible). If a litter failed to meet the sex ratio criteria (at least 3 pups/sex), the litter was not used for neurobehavioral or neuropathological evaluation. Following culling, a subset (Subset A) of 20 pups/sex/group was assigned to FOB (PND 4, 11, 21, 35, 45 and 60), acoustic startle response (PND 20 and 60), locomotor activity (PND 13, 17, 21 and 61) and learning and memory (PND 62). From this subset, 15 pups/sex/group were selected for

brain weight evaluations on PND 72; of these, 10 pups/sex/group were selected for neuropathological and morphometric evaluations on PND 72. A second subset (Subset B) of 20 pups/sex/group was selected for learning and memory tests (PND 22). A third subset (Subset C) of 15 pups/sex/group was selected for brain weight evaluations on PND 21; of these, 10 pups/sex/group were selected for neuropathological and morphometric evaluations on PND 21. Indicators of physical development (balanopreputial separation and vaginal patency) were evaluated for all F1 animals in Subset A. All F1 animals not selected for behavioural evaluations were euthanized and necropsied on PND 21. F1 animals selected for learning and memory assessment on PND 22 were necropsied following completion of these assessments.

Results: Dietary exposure to mancozeb from gestation day 6 through to weaning produced no test substance-related effects on clinical findings, FOB parameters, food consumption, gestation length, parturition or macroscopic findings at necropsy. Test substance-related effects were evident for maternal body weight gain, thyroid weight and thyroid histopathology at 30 mg/kg bw/d. Maternal body weight gain was reduced following the onset of treatment. The effect was statistically significant for gestation days 6–9 and 6-12. For gestation days 6-20, the decrease in body weight gain was 4.6%. However, the effect on body weight gain was influenced by an incidentally higher litter size in the 30 mg/kg bw/d group compared to the concurrent control group (16.0 vs. 14.7 pups/litter). When litter size was used as a covariate in the statistical analysis of body weight gain, the reduction at 30 mg/kg bw/d was highly significant. In addition, when the body weight gain data were normalized for litter size and gestation day 20 conceptus weights, the decrease in gain during gestation days 6-20 was 9.4%. Therefore, 30 mg/kg bw/day was shown to be an ideal high-exposure level based on the OECD test guideline criteria.

Summary of body weight change (g) during gestation

Gestation Days	Dose level of Mancozeb (mg/kg bw/day)			
	0	5	15	30
6-9	12	11	11	7*
6-12	27	25	24	20**
6-15	42	42	40	36
6-20	109	110	107	104

Statistically significant difference from control *P<0.05, **P<0.01

Summary of body weight change (g) during gestation at 30 mg/kg bw/day

Gestation Days	Dose level of Mancozeb (mg/kg bw/day)					
	Range Finding Study (N=15)			Developmental Neurotoxicity Study (N=25)		
	0	30	% Change	0	30	% Change
6-9	14	6**	-57.1	12	7*	-41.7
6-12	31	22**	-29.0	27	20**	-25.9
6-15	45	39	-13.3	42	36	-14.3

6-20	122	105**	-13.9	109	104	-4.6
Adjusted value for 6-20[a]	122	108.6	-11	109	98.8	-9.4

Statistically significant difference from control *P<0.05, **P<0.01

[a] Adjusted body weight gain normalized for differences in litter size and historical gestation day 20 conceptus weight across groups

A 30 mg/kg bw/d, absolute and relative mean thyroid weights were increased by 7.5% and 9.1%, respectively, and corresponded with an increased incidence of thyroid follicular cell hypertrophy; these changes were considered to be test substance-related. The maternal NOAEL was 15 mg/kg bw/d.

Summary of thyroid effects

	Dose level of Mancozeb (mg/kg bw/day)			
	0	5	15	30
Absolute thyroid weight (g)	0.0187	0.0192	0.0185	0.0201
Thyroid weight (g/100g final body weight)[b]	0.00638	0.007	0.006	0.00696
Thyroid hypertrophy, follicular cell - minimal	6/24	-	-	11/25

[b] Values for the 5 and 15 mg/kg bw/day groups expressed only to 3 decimal places.

There were no test substance-related effects on any of the F1 litter parameters including survival, clinical signs, FOB, growth, development, motor activity, startle response, learning and memory, brain morphometry and histopathology of the central and peripheral nervous systems. Therefore, the DNT NOAEL was 30 mg/kg bw/d, the highest dose tested.

Conclusions: Based on decreased body weight gain during gestation and an increased incidence of thyroid follicular cell hypertrophy at 30 mg/kg bw/d, the NOAEL for maternal systemic toxicity of mancozeb when administered orally in the diet was 15 mg/kg bw/d. There were no test substance-related effects on any of the F1 litter parameters investigated in this study; therefore, the DNT NOAEL was 30 mg/kg bw/d, the highest dose tested. Based on these findings and on the presence of ETU in pup plasma and in milk (investigated in the preliminary study), it can be concluded that mancozeb has been adequately tested for DNT and the results show that mancozeb has no developmental neurotoxicity.

5.2.10 Annex 2. Description of the Axelstad et al (2011) developmental neurotoxicity study

Methods: Two separate studies were conducted, a preliminary dose range-finder (6 dams/group) and a large dose response study (22 dams/group). Time-mated Wistar rats were gavaged once daily from GD7 to PND16 (not on day of delivery). No information was available on the purity of mancozeb (supplied by VWR-Bie & Bernsten, Herley, Denmark; lot no. 371-131c). Mancozeb was formulated in corn oil; no verification of dose concentration was performed. Dose levels for the preliminary study were 0, 200, 350 or 500 mg/kg bw/d. In the main study, dose levels were 0, 50, 100 or 150 mg/kg bw/d. The day after delivery pups were counted, sexed, weighed, checked for abnormalities and anogenital distance (AGD) measured. Body weights were measured on PND6, 13, 24, 31 and 45. Offspring were examined for the presence of nipples/areoleas on PND13 and

for motor activity on PND14, 17, and 23. On PND16 three males and two females per litter (when possible) were killed, weighed, and testes, epididymis, ventral prostate, ovaries, liver, adrenals and thyroid glands removed, weighed and processed for histopathological evaluation. On PND 24, 2-4 pups per litter were weaned and the dam and one male per litter were killed. Thyroids were removed weighed and processed for histopathology. In the dams the number of implantation sites were scored and for the male offspring the testes were weighed and processed for histopathology. Blood samples were collected for T_4 analysis: from dams on GD15 and PND24 and from pups on PND16 and PND24. Testosterone was measured from male pups killed on PND16. Behavioural tests in 2-7 month old offspring were: spatial learning in a radial arm maze, motor activity measurement and acoustic startle response. Adult offspring were killed at 8 months of age and ovaries and thyroids removed, weighed and prepared for histopathological examination.

Results: Doses of 150 mg/kg bw/d and above caused toxic effects in the dams. In the preliminary study, severe weight loss and hind limb paralysis occurred in all groups after a few days and doses were halved on GD12. All animals in the two highest dose groups and two in the low dose group were killed on GD14 due to continued toxic signs. In the main study, two high-dose dams were killed on GD16 and the high dose was lowered to 100 mg/kg bw/d. Maternal body weight and body weight gain was significantly lower than control in all dose groups throughout gestation. Body weight gain was generally higher than control in all groups throughout lactation but terminal body weight was significantly lower than control in the high-dose group.

There were no effects on gestation length, litter size, post implantation loss, neonatal death, gender distribution, AGD or nipple retention. Offspring body weight was lower compared to controls in the high dose group at birth and on PND6, and remained slightly (but not significantly) lower than control until PND45. In dams, T_4 was dose-dependently reduced in all treated groups on GD15. There was no effect on PND 24 and thyroid weights were also unaffected. In offspring no treatment-related effects on T4, thyroid gland weight or histopathology were seen.

Effects of Mancozeb on T4 and testosterone in dams and offspring

	Dose Mancozeb (mg/kg bw/day)			
Hormone measurement (nM)	Control	50	100	150/100
No. of samples	14-15	15-20	17-22	8-10
T_4 – dams GD15	41.4	32.7*	30.15*	26.26*
T_4 – dams PND24	24.8	21.2	19.6	23.14
T_4 – pups PND16	32.5	35.8	34.6	33.1
T_4 – pups PND24	18.5	19.8	22.1	24.3
Testosterone PND16	1.05	1.58	1.54	1.65

* Significantly different from control $p < 0.0001$

There were no effects on offspring organ weights on PND16 (testes, epididymis, prostate, ovaries, liver), PND24 (testes) or in young adults (ovaries) and no histopathological effects in ovaries and testes (other organ not examined). None of the behavioural tests showed any effect of mancozeb exposure.

Mancozeb at 150 mg/kg bw/d and above induced toxic effects in dams characterised by severe bodyweight loss and hind limb paralysis. The severity of the maternal toxicity was such that the dose had to be reduced to 100 mg/kg bw/d and some animals had to be terminated prematurely. Increased maternal T_4 levels at 50 mg/kg bw/d and above did not affect offspring T_4 levels, thyroid weights or histopathology on PND 16 and no effects on reproductive organ weights or behaviour in adult offspring. Overall, no developmental toxicity was seen in this study up to a dose of 100/150 mg/kg bw/d. Thyroid toxicity (increased T_4 levels) was observed in the maternal animals from the lowest dose of 50 mg/kg bw/d. These results indicate that in rats, maternal hypothyroxinemia during gestation does not necessarily lead to hyperactivity or reduced learning abilities in the

offspring. Overall, a maternal LOAEL of 50 mg/kg bw/d can be identified from this study and a NOAEL > 100/150 mg/kg bw/d can be identified for developmental toxicity.
It is noted that the maternal toxicity reported in this study was not consistent with that observed in other developmental toxicity studies. In regulatory guideline studies, pregnant rats have been shown to tolerate 160 mg/kg bw/d (Edwards, 2015c) with only a transient effect on body weight. Only the high dose level of 360 mg/kg bw/d (Tesh, 1988) induced significant maternal toxicity, including transient, hindlimb paralysis.

5.2.11Annex 3. Description of OECT TG 443 EOGRTS ETU study (with DNT cohort) from the EU RAR (Marty et al 2013b in EU RAR)

Methods :In an extended one generation toxicity study, ETU was administered in the diet to groups of 27 male and 27 female Sprague Dawley rats at nominal dose levels of 0, ~0.2, 2.0 and 10 mg kg bw/d (0, 2.8, 28, 140 ppm) for approximately four weeks prior to breeding, and continuing through breeding (two weeks). After breeding, P1 males continued on the test diets for an additional 5-7 weeks (11 weeks total exposure) and P1 females continued on the test diets through gestation and lactation (10-12 weeks total exposure). Dose levels were selected on the basis of a range-dosing study in which groups of 12 male and 12 female SD rats were fed diets supplying 0 (control), 0.2, 2.0, and 10 mg/kg/d ETU. Males were exposed for at least two weeks prior to breeding and continuing throughout breeding and post-breeding for approximately 8 weeks of exposure. The females were exposed for two weeks prior to breeding, continuing through breeding (up to two weeks), gestation (three weeks), and lactation (three weeks). In this preliminary study, palsma kinetics of ETU were also assessed in parental animals and F1 offspring. In the main study, F1 offspring were divided into Cohorts 1A, 1B, 2A, and 2B at weaning (postnatal day (PND) 21).

Cohort 1A (26/sex/dose) was used to evaluate reproductive toxicity, which included estrous cycle evaluation and post-mortem examination of reproductive organs, sperm assessment, and ovarian follicle counts on PND 90. This group was also used to assess general and thyroid toxicity, which included clinical chemistry/haematology parameters, thyroid hormone assessment, and urinalysis. Post-mortem evaluations in Cohort 1A (PND 90) also included gross pathology, organ weights, and histopathology on a wide range of tissues, including thyroids.

Cohort 1B animals (26/sex/dose) were known as the endocrine group and designated to clarify any equivocal responses seen in the Cohort 1A animals. This group was also used to assess general and thyroid toxicity, which included thyroid hormone assessments. Post-mortem evaluations in Cohort 1B (PND 120) included gross pathology and organ weights with a primary focus on tissues affected in Cohort 1A, including thyroids.
The Cohort 2A and 2B animals (22/sex/dose) were used to assess potential developmental neurotoxicity (DNT). Cohort 2A (12/sex/dose) was used for DNT assessments, which included functional observational battery (FOB), motor activity, and acoustic startle response (ASR). On PND 78, Cohort 2A F1 animals were perfused for central nervous system (CNS) and peripheral nerve neuropathology evaluation and brain morphometry.
Cohort 2B (10/sex/dose) underwent necropsy on PND 22, which included brain weight collection and immersion fixation of tissues for examination of neuropathology.

Weanlings not assigned to Cohorts 1-2 were considered "unselected" weanlings. On PND 22, these unselected weanlings (15/sex/dose) were euthanized for assessment of systemic toxicity, which included thyroid hormone assessment, selected organ weights, and post-mortem examinations (gross pathology and histopathology). In addition, selected pups culled on PND 4 were used to assess thyroid hormone levels.

Results: Nominal dose levels (mg/kg bw/d) were achieved across both P1 and F1 generations; however, during some study intervals (e.g., PND 35-56), F1 offspring had greater ETU intake per kg body weight than P1 males and prebreeding females. In the range-finding study, plasma concentrations of ETU in all groups were dose-proportional (linear) across all dose levels. Plasma AUC24h values on GD 20 were also dose proportional (linear) across all ETU-treated dose groups. There were no observed sex- or lactation-related differences in ETU kinetics. In LD 4 culled pups, mean plasma ETU concentrations were approximately 22% of dam plasma levels. Plasma concentrations of ETU in LD 21 pups were approximately 65% of dam levels. The higher pup-to-dam ETU plasma concentration ratio at LD 21 was likely due to pup consumption of ETU-containing diets, in addition to suckling. In the parental generation, high-dose ETU females had decreased body weight (7-8%) on lactation days (LD) 4 and 7, which coincided with decreased feed consumption (5-10%) and decreased F1 pup body weights on PND 14 and 21 (decreased by 11-12% and 8-9%, respectively). The F1 body weights in the high-dose males were significantly different from the controls from PND 21 through cohort termination or until PND 112 (7.4% decrease in body weight in Cohort 1B). The F1 high-dose female body weights recovered to control values by ~ PND 56.

ETU had no effect on male or female reproductive performance. Sperm numbers and mobility and estrous cycle length were unaffected. There were no effects on mean number of pups delivered per dam, pup survival rates of liveborn and stillborn pups or sex ratio.

Summary of female reproduction and delivery data

Parental generation	**P1**			
Dose [ppm]	**0**	**2.8**	**28**	**140**
Animals per dose	27	27	27	27
Female fertility				
- placed with males	27	27	27	27
- mated [n]	27	27	27	27
- mating index [%]	100	100	100	100
- pregnant [n]	26	26	26	26
- **Fertility index** [%]	96.3	96.3	96.3	96.3
Pre coital interval[days]	2.3±1.1	3.4±2.4	2.3±1.1	2.8±1.2
Duration of gestation [days]	21.5±0.5	21.5±0.5	21.7±0.5	21.8±0.5
Post implantation loss				
- dto per litter[mean %]	8.86±10.13	6.13±6.19	6.60±10.11	9.67±10.20
Females with liveborn	26	26	26	26
- **Gestation index** [%]	100	100	100	100
Pups delivered [n]	348	363	365	357
- per dam [mean n]	13.4	14.0	14.0	13.7

- liveborn [n]	343	360	357	351
- **Live birth index** [%]	98.6	99.2	97.8	98.3

Treatment-related thyroid effects included significant changes in thyroid hormone profile, thyroid weights, and thyroid histopathology (follicular cell hyperplasia) at doses ≥ 2 mg/kg bw/d ETU in both males and females at multiple life stages. There was a significant, treatment-related increase (65%) in serum TSH in high-dose PND 4 culled pups. T4 was decreased by 28% at this dose level. There were significant decreases in serum T4 in both male and female weanlings (PND 21) at the top dose. Serum T4 also was significantly decreased at 28 ppm in female weanlings. There were corresponding significant increases in TSH levels in both male and female weanlings at the top dose. Females also had significant increases in TSH in the 28 ppm group. Both male and female PND 22 weanlings from the 140 ppm group had treatment-related higher absolute and relative thyroid gland weights, which were associated with diffuse hyperplasia and hypertrophy of thyroid follicular cells. Very slight diffuse follicular cell hypertrophy of the thyroid gland was also present in 3/15 males and 3/15 females PND 22 weanlings from the 28 ppm group.

Serum T4 and TSH levels in male and female PND 4 Pups

Dose (ppm)	**Males and Females**			
	T4 (µg/dL)	% Change	TSH (ng/mL)	% Change
0	0.82	NA	0.89	NA
2.8	0.48*	-41	0.92	-3
28	0.72	-12	1.05	+18
140	**0.59**	**-28**	**1.47***	**+65**

NA = not applicable, * statistically different from control mean by Wilcoxon's test, Bold type indicates the effects were interpreted to be treatment related

Serum T4 and TSH Levels in male and female PND 22 weanlings

Dose (ppm)	**Males**				**Females**			
	T4 (µg/dL)	**% change**	**TSH (ng/mL)**	**% change**	**T4 (µg/dL)**	**% change**	**TSH (ng/mL)**	**% change**
0	3.73	NA	0.95	NA	3.11	NA	0.87	NA
2.8	3.71	-0.5	0.89	-6	3.13	+0.6	1.03	+18
28	3.23	-13	1.14	+20	**2.35***	**-24**	**1.32$**	**+52**
140	**1.76***	**-53**	**2.68$**	**+182**	**1.57***	**-50**	**2.40$**	**+176**

NA = not applicable, * statistically different from control mean by Dunnett's test, $ statistically different from control mean by Wilcoxon's test. Bold type indicates the effects were interpreted to be treatment related

No treatment-related effects on sexual maturation were observed in male or female F1 pups. Body weight at the time of vaginal opening was significantly lower (7%) in the

high-dose females but this was indicative of the ETU-induced reduced growth rate as age at vaginal opening was not altered. Puberty onset was delayed by 1.9 days in the high dose group males, but this effect was considered to be related to body weight decrements and slightly delayed growth as confirmed by the observation that juvenile F1 male pups in the high-dose group weighed 7% less than controls at PND 28, and 8% less than controls on PND 35 and 42. There were no significant, treatment-related differences in age at preputial separation in male offspring, absolute or relative anogenital distance and nipple/areolae retention in either male or female offspring.

Sexual maturation of F1 pups

Sex	**Male**		**Female**	
	Age days	Body weight [g]	Age days	Body weight [g]
Dose				
0	43.5 ± 1.7	245.4 ± 19.4	31.7 ± 1.2	112.4 ± 9.8
2.8	44.4 ± 2.1	243.2 ± 20.1	31.9 ± 0.9	109.9 ± 9.0
28	44.5 ± 2.7	242.4 ± 17.5	31.8 ± 1.0	111.1 ± 9.5
140	45.4* ± 1.9	239.5 ± 20.4	31.9 ± 0.9	104.1* ± 7.1

* statistically different from control mean by Wilcoxon's test

There were no treatment-related effects on clinical-chemistry and haematological parameters at any dose level in P1 adults. High-dose P1 males had significant decreases in serum concentrations of T4 (67%) and a coincident significant increase in serum TSH levels (326%). At the mid dose, P1 males had a significant decrease in serum T4 levels (23%), but TSH levels were not significantly altered. For high-dose P1 females on LD 22, there was a significant decrease (76%) in serum T4 levels and a corresponding significant increase (323%) in serum TSH concentrations. At the mid dose, P1 females had a significant decrease in serum T4 levels (36%), but TSH levels were not significantly altered. High dose P1 animals showed increased absolute and relative thyroid weights.

High- and mid-dose P1 animals had statistically significant lower absolute and relative thymus weights. The decreased thymus weights were associated with atrophy of lymphoid tissue at the high dose only. Effects on the thymus were not seen in the F1 offspring.

Histopathology of the thyroid in parental animals revealed the presence of follicular cell hyperplasia at the mid- and high-dose. Slight follicular cell hypertrophy was also seen mainly at the top dose. Very slight follicular cell hypertrophy was noted at the low dose in P1 males and F1 cohort 1A males. Given the very mild severity of this finding in the low dose males and the absence of effects on thyroid hormone levels at this dose, the very slight follicular cell atrophy of the thyroid at 0.2 mg/kg bw/d was considered not to be adverse. Histopathology of the pituitary in parental animals revealed a significantly increased incidence of slight hypertrophy of individual cells of the pars distalis at the high dose. Slight hypertrophy was also seen at a very low incidence at the low and mid dose in males only of the P1 generation and F1 Cohort 1A generation. Given the low incidence of this finding at the low and mid dose, it was considered not to be adverse. Histopathology of the liver in parental animals showed slight hepatocyte vacuolation in high dose males only of the P1 generation.

Incidence of selected histopathological lesions in parental rats

Dose [mg/kg]	**0**	**3.8**	**38**	**190**	**0**	**2.8**	**28**	**140**
Sex	**male**				**female**			
Animals in group	27	27	27	27	27	27	27	27
Thyroid gland **#** examine	27	27	27	27	27	27	27	27
- hyperplasia, follicular cell, diffuse								
-very slight	0	0	**14**	**4**	0	0	**7**	**1**
-slight	0	0	**1**	**23**	0	0	0	**26**
- hyperplasia, nodular, follicular cell, focal								
-slight	0	0	0	**1**	0	0	0	**1**
- hypertrophy, follicular cell, diffuse								
-very slight	4	**20**	**21**	**4**	2	2	**12**	1
-slight	0	0	**1**	**23**	0	0	0	**26**
- adenoma, follicular cell, benign, primary	0	0	0	**2**	0	0	0	0
Pituitary **#** examined	27	27	27	27	27	27	27	27
- hypertrophy, pars distalis, individual cells								
-very slight	0	0	0	0	1	2	4	3
-slight	0	**4**	**5**	**24**	0	0	3	**22**
Liver **#** examined	27	27	27	27	27	27	27	27
- vacuolization, consistent with fatty change, hepatocyte, individual cells, multifocal								
-very slight	23	24	26	18	3	0	0	2
-slight	0	2	0	**8**	0	0	0	0

Bolded values interpreted to be treatment related

Incidence of selected histopathological lesions in Cohort 1A rats – PND90

Dose [mg/kg]	**0**	**2.8**	**28**	**140**	**0**	**2.8**	**28**	**140**
Sex	**male**				**female**			
Animals in group	26	26	26	26	26	26	26	26

Thyroid gland	**#** examine	26	26	26	26	26	26	26	26
- hyperplasia, follicular cell, diffuse									
-very slight		0	0	**11**	**4**	0	0	0	**12**
-slight		0	0	**3**	**22**	0	0	0	**9**
- hyperplasia, nodular, follicular cell, focal									
-slight		0	0	0	**1**	0	0	0	0
- hypertrophy, follicular cell, diffuse									
-very slight		4	**15**	**12**	4	0	0	**3**	**12**
-slight		0	0	**3**	**22**	0	0	0	**9**
- adenoma, follicular cell, benign, primary		0	0	0	**1**	0	0	0	0
Pituitary	**#** examined	26	26	26	26	26	26	26	26
- hypertrophy, pars distalis, individual cells									
-very slight		3	**9**	**9**	**6**	0	0	0	0
-slight		0	0	**6**	**13**	0	0	0	0

Bolded values interpreted to be treatment related

There was no evidence of treatment-related reproductive toxicity or effects on the estrogen- or androgen-related endocrine pathways at any dose of ETU. The *a priori* triggers for producing a second generation were not met; therefore, the F1 animals were not mated in this study.

For developmental neurotoxicity, there were no effects on brain weights in the Cohort 2B animals (PND 22); however, there was a decrease in overall brain size (6-7% decrease in absolute brain weight) at the highest dose of ETU in the Cohort 2A females (PND 78) and Cohort 1A males (PND 90). The relative brain weight in Cohort 1A males on PND 90 was not statistically significantly different from controls (slightly increased) and for females in Cohort 2A the individual values were generally within the range of concurrent controls (2 values 2.155 and 2.171 marginally below lowest control value of 2.174).

Group mean body weights and brain weights

Males				
	0 (Control)		140 ppm	
	Final body weight	Brain weight (g)	Final body weight	Brain weight (g)
Cohort 2B (PND 22)	56.8	1.543	52.0	1.527
Unselected (PND 22)	55.2	1.496	54.3	1.487
Cohort 2A (PND 78)	430.2	2.491	380.0	2.352

Cohort 1A (PND90)	498.4	2.131	447*	2.017*
Females				
	Final body weight	Brain weight (g)	Final body weight	Brain weight (g)
Cohort 2B (PND 22)	54.8	1.498	50.2	1.458
Unselected (PND 22)	55.0	1.483	52.7	1.465
Cohort 2A (PND 78)	251.5	2.385	243.0	2.227*
Cohort 1A (PND90)	262.1	1.938	252.7	1.877

The lower brain weight in the Cohort 2A animals was accompanied by decreases in overall brain macroscopic and microscopic measurements (1-4% decrease across all gross brain measurements: ≤ 5% change in microscopic measurements), but there were no differences in these parameters at ≤ 28 ppm. The differences in brain weight and measurements at 140 ppm indicate a slightly smaller brain size with no specific brain region/layer showing a statistically significant effect.

At 140 ppm there were effects on pup body weight from PND 14 which persisted in males until PND 112 (7.4% lower in Cohort 1B). In females body weights were not statistically significantly different from PND 56 but remained slightly lower than control. Overall, the data support the conclusion that growth at 140 ppm was reduced during late lactation, juvenile and early adult periods. Brain weight and body weight show a positive correlation and absolute organ weight is never an optimal endpoint for the evaluation of organ weight changes in the presence of body weight differences between the groups. The differences in brain weight (6-7%) were relatively small compared to the effects on body weight (up to 10%), many individual values were within the range of concurrent controls, and the morphometric analysis indicated that the brain was slightly small overall, rather than decreased in any specific region/layer. Consequently, in the absence of any effects on neurobehavioural endpoints or neuropathology, the differences in brain weight at 140 ppm ETU are considered to reflect the reduced growth during late lactation, juvenile and early adult periods and not to be evidence of specific developmental neurotoxicity.

Conclusions

In this guideline extended one-generation study, ETU caused no effects on reproductive toxicity or developmental neurotoxicity up to the top dose of 10 mg/kg bw/d. There was however parental toxicity and offspring toxicity from a dose of 2 mg/kg bw/d, manifested as effects on body weight (in adults and pups), thymus (decreased weight and atrophy of lymphoid tissue in adults), pituitary (hypertrophy in adults) and liver (vacuolation in adults) at the top dose and thyroid toxicity (hypertrophy in adults) at the mid and high dose. Overall therefore a NOAEL of 10 mg/kg bw/d is identified for reproductive toxicity and developmental neurotoxicity and a NOAEL of 0.2 mg/kg bw/d is identified for parental toxicity and offspring toxicity (Marty et al., 2013b).

5.3 PERCHLORATE CASE STUDY

5.3.1 Summary

The effects of perchlorate have been investigated in humans on many occasions, including in studies from clinical and occupational exposures, studies with healthy volunteers and epidemiological studies. Results from a number of these publications are presented in this case study with the aim of elucidating the effects of the chemical on iodide uptake, thyroid function and its impact on the hypothalamus-pituitary-thyroid (HPT) axis in humans. From all these studies, a relatively consistent picture emerges: in healthy humans, an impact on thyroid function will only likely arise from prolonged

exposures to levels higher than 0.4 mg perchlorate/kg b.w. per day. However, this statement needs to be interpreted against the background of widespread iodine deficiency and co-exposure to other iodide-uptake inhibitors, such as thiocyanate (from cigarette smoking) occurs which may exacerbate the effects of perchlorate. However, due to the limitations highlighted with epidemiological studies and the assessment of real exposures in populations, it is unclear whether lower dose could cause adverse effects in pregnant women, infants, children and people with low iodine intake or pre-existing thyroid dysfunction.

In relation to evidence from animal studies, most of the data presented in this case study are from the rat model, even though studies have also been carried out in rabbits and mice. Sub-chronic and neurodevelopmental studies in the rat have shown that the rat is indeed a highly sensitive species, with changes in thyroid hormone levels at doses of 0.1 mg/kg b.w. per day.

As the aim of this study was to compare the sensitivity to perchlorate of the rat and humans, a large section is dedicated to comparing the outcome of short-term and long term exposures between the two species. One relevant point from this analysis is that the most vulnerable life stage in the rat is during gestation and postnatally. Even though the sensitivity of this life stage was compared to adult humans, a comparison to corresponding pregnant women or women post-partum is difficult to conduct due to a lack of relevant data.

At the end of this case-study a section on approaches to the study of thyroid disruption from the ecotoxicology arena has been included. Here we propose a number of models using invertebrates, fish and fish embryos for consideration as potential alternatives to the rat as systems to investigate human thyroid disruption.

5.3.2 Scope and aims of this case study

The proper delivery of thyroid hormone to target cells and tissues both during development and in adulthood is dependent upon a variety of steps that are unique to this endocrine system. The aspect of the thyroid disruption to be discussed in this case study is the sensitivity of the rat model to iodide-uptake inhibitors in relation to humans, and whether the rat is an appropriate model for the identification of hazards and risks from sodium-iodide symporters to the human population. More specifically, the following questions are discussed, both in relation to their scientific implications and their regulatory significance and impact:

- How do humans and rats compare in relation to their response to short and long term exposures to perchlorate?
- If taking into account vulnerable subpopulations, such as pregnant women, newborns, infants, people with insufficient dietary iodine (defined by WHO) and people exposed to other chemicals that interfere with iodide uptake (eg: chlorate, nitrate, and thiocyanates), could the rat model be more representative of the effects in humans?
- Could models widely employed to assess the impacts of thyroid disruptors in ecotoxicology be used for hazard identification?

In order to address these points, the present case study includes information from perchlorate studies found in the open literature, as well as data from the NRC (NRC, 2005) and EFSA scientific opinion paper on the risks of perchlorate to public health (EFSA, 2014). However, this paperis not meant to be an exhaustive substance evaluation, so only data relevant to the questions mentioned above is presented here.

5.3.3 Perchlorate and its uses

Perchlorate is an oxy-anion of chlorine which, when combined with cations like sodium, potassium, ammonium, forms perchlorate salts, such as sodium perchlorate, potassium perchlorate and ammonium perchlorate. Perchlorate occurs in the environment from natural and anthropogenic sources. The most common use of perchlorate salts is as

oxidising agents in the manufacture of solid rocket propellants, explosives, pyrotechnics, grenades, signal flares and matches (Sijimil and Mohan, 2014).

In terms of human exposure, different sources of contamination have been identified. These include the use of fertilisers of natural origin from countries with large natural deposits (such as the Atacama desert in Chile), industrial emissions of perchlorate into the environment, the natural formation of perchlorate in the atmosphere and surface water, and the formation of perchlorate during the degradation of chlorine-based products, such as sodium or calcium hypochlorite. In Europe, the use of natural fertilisers such as Chilean nitrate may lead to substantial concentrations in fruit and vegetables. Similarly, plant irrigation with perchlorate-contaminated groundwater can contribute to the accumulation of perchlorate in fruit and vegetables. Water disinfection with chlorine-based biocidal products, potentially degrading to perchlorate, could be another notable source of contamination for drinking water and food. Perchlorate has been reported to occur in a wide range of foods, including vegetables, fruit, milk and dairy products, juice, beer, wine and bottled water (EFSA, 2014). An important issue to bear in mind is the fact that, in 2005, the FDA granted approval to using perchlorate as an anti-static agent in dry good plastic packaging. The chemical compound was already approved as an additive in sealing gaskets for food containers. Alarmingly, a report by Murray et al (2008) indicated that, unlike previously assumed, perchlorate is able to migrate from plastics into food and that almost 75% of all food types are contaminated with perchlorate.

5.3.4 Mechanism of action of perchlorate

The main toxic effect of perchlorate (both in humans and experimental animals) involves alterations in thyroid hormone levels, as a result of its competitive inhibition of thyroidal uptake of iodide into the thyroid follicular cells.

The main function of the thyroid is to produce the thyroid hormones triiodothyronine (T3) and thyroxine (T4). One of the crucial steps of hormone synthesis in the thyroid involves the uptake of iodide into the thyroid follicular cells by the sodium iodide symporter (NIS) present in the basolateral membrane of the thyroid follicular cells. The perchlorate anion interacts with NIS competing with iodide for cell uptake. The perchlorate anion is then transported further into the thyroid lumen before it diffuses passively out of the thyroid gland into the blood supply and is eliminated in urine. The NIS has been identified at the molecular level of some extrathyroidal tissues, such as the lactating mammary gland, the placenta, the salivary gland and gastric and intestinal mucosae, areas where the physiological functions of iodide transport have yet to be elucidated (reviewed in Leung et al., 2010). In breast tissue, the NIS is thought to play a role in the transport of iodine into breast milk. In addition to the direct effect in NIS, perchlorate is also able to reduce the levels of iodine in the thyroid by increasing free iodine loss from the organ.

The effects of perchlorate on iodine availability should be seen in the context of other NIS inhibitors. These include thiocyanate, a metabolite of cyanide that is produced as a by-product of cigarette smoke and found in a large variety of foods, and nitrate, which is produced naturally. Comparatively, perchlorate is a very potent inhibitor of the NIS; its effects are 15-fold greater than thiocyanate, 30-fold compared to iodide, and 240-fold compared to nitrate. Nonetheless, because exposure to thiocyanate and nitrate is ubiquitous, the additive effects on iodide uptake may be important when assessing iodine availability. Some have urged that the detection, concentrations and potencies of these other NIS inhibitors also be considered in studies reporting environmental perchlorate exposure, which were not assessed in the large U.S. population study reporting the adverse effects of perchlorate exposure on thyroid function in women (see section on "Observations from human studies with relevance to thyroid disruption").

Severe and sustained inhibition of iodine uptake in the thyroid following exposure to perchlorate can limit the availability of iodine required for the production of thyroid hormones T3 and T4, which could cause depletion of thyroid stores of these hormones and lower hormone serum levels. Decreases in circulating levels of thyroid hormones will trigger the hypothalamic-pituitary-thyroid (HPT) feedback pathway, resulting in an

increase in secretion of Thyroid Stimulating Hormone (TSH). TSH stimulates thyroid iodine uptake and the production and secretion of the thyroid hormones T3 and T4, and promotes growth of thyroid follicle cells. Persistent stimulation of the thyroid gland by elevated levels of TSH results in increases in thyroid gland size and weight (goitre), decreased colloid, hypertrophy and hyperplasia of thyroid follicle cells and thyroid tumours in rats (Fisher et al., 2012). Furthermore, thyroid hormones are essential for normal fetal growth and differentiation of many organs, especially in pre- and post- natal central nervous system and brain development. The fetus is dependent on maternal thyroid hormones until it can produce its own at approximately 16-20 weeks of gestation. Hence, during pregnancy, women with low T4 levels could be at risk of producing children with neuronal disabilities.

5.3.5 Observations from human studies with relevance to thyroid disruption

Here, we present a summary of the most relevant findings in terms of the human effects of perchlorate. This is not intended as an extensive review of the literature, but only to elucidate points of current debate.

Potassium perchlorate was introduced as a form of therapy for thyrotoxicosis (overactive thyroid with increased production of thyroxin) in the 1950s. Several studies were published in the 1970s and 1980s analysing the side effects of perchlorate used to treat hyperthyroidism. A report by Wenzel and Lente (1984) presented the case of 18 patients suffering from Graves disease who were treated with 9mg ion/kg b.w. per day until the serum thyroid hormone levels were reduced. This was followed by a maintenance period of 12 months, when patients were treated with 0.4 – 1.2 mg perchlorate /kg b.w. per day for 12 months. During the maintenance therapy periods, the patients showed normal T3 and T4 levels and normal TSH-receptor stimulating antibodies and no side effects were reported in the study.

In healthy adults, several reports have shown that exposure to levels of perchlorate ranging from 0.007 to 9mg ion/kg b.w. per day for periods between 14 days and 6 months did not result in significant changes in hormone levels or thyroid function (Brabant et al., 1992; Lawrence et al., 2000; Lawrence et al., 2001; Braveman et al., 2006). Endpoints analysed included serum total and free T4, total T3, thyroglobulin and TSH levels, and thyroid iodine uptake. In two of the studies (Lawrence et al., 2000 and Lawrence et al., 2001) there was a significant increase in relation to the baseline of thyroid hormones levels after treatment discontinuation, indicating an adaptive change of the iodine uptake in the presence of the inhibitory effects of perchlorate. However, a key issue here is that the levels of iodine in these different study populations was different, which affects the severity of the impacts seen in response to perchlorate.

Of note is also the publication by Greer (2002) which reported the effect of perchlorate on the thyroid uptake of radiolabelled iodine in human volunteers in a study aimed at establishing the dose response in humans for perchlorate inhibition of thyroidal iodide uptake. For that, Greer and colleagues administered oral doses of perchlorate between 0.007 and 0.5 mg/kg b.w. per day for 14 consecutive days. Iodine uptake statistically significantly decreased at ≥ 0.02 mg perchlorate/kg b.w. per day during the treatment period, reaching a mean inhibition of 67% at 0.5 mg/kg b.w. per day, whereas no statistically significant differences from the baseline were observed on the uptake on day 15 (post-treatment). No statistically significant changes were observed in total T4, free T4, total T3 or TSH during the study period in any treatment group in comparison with the baseline levels, but these findings may not be surprising considering that the goal of this study was to determine the dose at which perchlorate reduced iodide uptake, and that T_4 levels were predicted to remain unaffected.

Retrospective epidemiological studies at the general population level, including sensitive subjects such as pregnant women and infants, showed varying results, some of them indicating changes in thyroid hormone levels in populations living in areas with known perchlorate contamination in drinking water (> 5 µg/L), others failing to demonstrate associations.

Studies of infants and newborns exposed via mothers drinking tap water during the gestation period (Lamm and Doemland, 1999; Chang et al., 2003, Brechner et al., 2000, Buffer et al., 2006 and Pearce et al., 2010) did not find significant correlations between perchlorate levels and thyroid function. Outcomes evaluated included neonatal congenital hypothyroidism, neonatal thyroid parameters (T4 and TSH) and potential indicators of neurodevelopmental effects (attention deficit hyperactivity disorder and school performance).

Associations between increasing concentrations in urine and a decrease in total T4 and increasing TSH in pregnant women have been seen (Steinmaus et al., 2016). Of note, Steinmaus et al. (2016) measured individual levels of perchlorate. An earlier study by Tellez et al. (2005) in which individual perchlorate levels were not measured did not see such an association (Tellez et al., 2005).Taken together, the changes reported in these epidemiological studies were of limited magnitude. Importantly, positive associations were mostly observed when potential exposure to perchlorate occurred in the presence of other risk factors, such as low dietary iodine intake, or exposure to other iodide uptake inhibitors, such as thiocyanate, via tobacco smoke (Steinmaus et al., 2013).

However, it is important to bear in mind that many of these studies are of limited use for the evaluation of risks, because of the limited, or non-existent, information on individual exposure levels. For example, the study by Lamm and Doemland (1999) analysed neonate incidences of hypothyroidism in 6 counties in the states of California and Nevada, all known to have high levels of perchlorate in water. Concentrations varied between counties, but the authors did not measure individual levels of perchlorate, but instead correlated the cases of congenital hypothyroidism with the concentrations of perchlorate in drinking water in the different counties. The use of city or state of residence as a surrogate for exposure invalidates the study. In order to identify a relationship between perchlorate and hypothyroidism, it would have been essential to determine the individual levels of the chemical in the affected individuals. Another issue with these studies is the general natural high variability of the thyroid hormone levels, and the presence of possible confounding factors that are not taken into account in the analysis (e.g. the potential effect of altitude on changes in thyroid hormone levels).

On the basis of the body of evidence available from clinical studies, adult volunteers and epidemiological studies, NRC (NRC, 2005) and CONTAM (EFSA, 2014) concluded that hypothyroidism will likely only arise from a prolonged exposure to more than 0.4 mg perchlorate/kg b.w. per day in normal adults. At the same time, both NRC and EFSA stated that a lower, not further specified, dose could be sufficient to cause adverse effects in pregnant women, infants, children and people with low iodine intake or pre-existing thyroid dysfunction.

This is significant, considering that iodine deficiency is a substantial, global issue, with an estimated 2.2 million people (38% of the world's population) living in iodine-deficient areas. A report by US EPA estimates that at least 20% of pregnant women are already iodine deficient, resulting in T4 levels that put the fetuses' developing brains at risk. For this population of pregnant women, it can be anticipated that any perchlorate exposure is likely to result in an even greater risk of impaired brain development in their children and potentially a lifetime of behavioral and learning difficulties. However, to date, no direct evidence is available to substantiate this possibility.

5.3.6 Observations from experimental studies

5.3.6.1 Experimental evidence – mammalian assays (rats)

In a study by Siglin et al. (2000), Sprague-Dawley rats were exposed to ammonium perchlorate via the drinking water at doses ranging from 0.01 to 10 mg/kg b.w. per day for 90 days, followed by 30 days recovery period. After exposure, significant decreases in serum T3 and T4 were noted for both sexes at doses higher than 0.01 mg/kg b.w. per day. Significant increases were also seen in serum TSH in males at doses higher than 0.2

mg/kg b.w. per day and in females at 10 mg/kg b.w. per day. Increases in thyroid weight, thyroid follicular cell hypertrophy and colloid depletion were also noted in both sexes at the highest dose tested. Following the 30-day recovery period, significant decreases in serum T4 were found for males at all doses. In females, serum TSH was significantly increased at all doses and serum T3 was significantly decreased at the highest dose. Seeing as even the lowest dose induced thyroid hormone changes, the overall LOAEL for this study was considered to be 0.01 mg/kg b.w. per day. Similar results were reported by Stoker et al. (2006) who also saw dose-dependent decrease in serum T4 (125, 250 and 500 mg/kg doses), increase in TSH (doses: 125, 250 and 500 mg/kg) and alterations in thyroid histology (at doses 62.5, 125, 250, and 500 mg/kg), namely follicular cell hypertrophy and decreased colloid area.

Neurodevelopment effects

In a study by York et al. (2004) investigating developmental neurotoxicity, female Sprague-Dawley rats were administered ammonium perchlorate continuously from GD0 to PND 10 at concentrations ranging from 0.1 to 10.0 mg/kg b.w. per day. An increase in thickness of the corpus callosum was noted in pups at the highest dose. An increase in incidence of thyroid follicular epithelium hypertrophy and hyperplasia and a reduction in follicle size was seen for female pups at doses higher than 3 mg/kg b.w. per day and males pups at 10.0 mg/kg b.w. per day, making histopathological effects the most sensitive endpoint. Decreases in thyroid hormone levels and increases in TSH levels were also noted at the highest dose tested. In another publication York and colleagues (2005) reported significant increases in the linear dimensions of a number of brain regions in male pups at all doses and a reduction in the size of certain brain regions in female pups. However, no effects on male rat brain weights or neuropathology were noted.

In a later study by Gilbert and Sui (2008), female Long-Evans rats were exposed to ammonium perchlorate at 4.5, 44.2 and 140.3 mg/kg b.w. per day from GD6 to PND 30. Serum TSH levels in pups were significantly increased at 4.5 and 44.4 mg/kg b.w. per day. Significant decreases in serum T3 and T4 were noted at the highest dose were noted in pups. In adult male offspring (five to nine months old), significant dose-dependent reductions in baseline synaptic transmission were observed in hippocampal field potentials in all three doses. All serum hormone concentrations returned to normal in adulthood and no behavioural alterations were observed in hippocampal-based learning tasks. However, there was a significant reduction in baseline synaptic transmission in hippocampal field potential that persisted in the adult offspring suggesting that developmental exposures to perchlorate may result in permanent alterations in brain function.

In summary, the studies described above demonstrate that perchlorate perturbs the HPT axis in the adult rat, pregnant and lactating dams and PND 22 pups of both sexes. This means that when serum TSH levels were elevated, the corresponding serum thyroid hormones were either decreased or maintained similar to control levels of thyroid hormones.

Nevertheless, NRC (2005) and EFSA (2014) highlighted concerns regarding the interpretations of results of developmental neurotoxicity studies with perchlorate due to design and methodological problems and to the lack of sensitivity of the neurobehavioral tests for the detection of subtle changes in motor or cognitive function resulting from moderate reductions in thyroid hormone levels. Based on the available data and the limitations listed, the recent CONTAM panel (EFSA, 2014) concluded that 'it is not possible to determine an association between exposure to perchlorate and development neurotoxicity from the studies in rats'.

5.3.6.2 Interspecies differences in sensitivity to perturbation of thyroid homeostasis – comparison between rat and humans

While the data strongly supports the notion that both humans and rats are affected by perchlorate in terms of thyroid dysfunction in both short and long term exposures, the evidence also shows that the two species exhibit differences in terms of thyroid hormone

and HPT axis responses to the inhibition of iodide uptake caused by the chemical. Two reports, Lewandowsky at al., 2004 and Fisher et al., 2012 have addressed this issue by comparing the outcomes of a number of rat and human studies. The results from Lewandowsky et al., 2004 are summarised here:

In the rat, thyroidal iodide uptake is markedly depressed after an acute intravenous dose of perchlorate, within the span of a couple of hours (Yu et al., 2002). A similar response is seen in adult humans within two days of exposure (Greer et al., 2002), however in this case the exposure was from drinking water. No other data is available for acute effects of perchlorate in humans.

The situation is markedly different with longer perchlorate exposures. In this case, the differences between the rat and humans in terms of iodide uptake are more substantial. After administration of perchlorate in drinking water for 14 – 23 days, rats at various life stages (male, pregnant female and postnatal female dam) have less uptake inhibition and, in some cases, have iodide uptakes which are higher than pre-dosing baseline levels (Yu et al., 2002). In contrast, the human response after 14 days of perchlorate exposure is similar to the acute responses after two days (Lawrence et al., 2000; Greer et al., 2002) with a significant decrease in iodide uptake.

A similar pattern is reported for thyroid hormone levels. Lewandowski and colleagues' (2004) analysis showed that male and female rats exhibit a clear upward trend in serum TSH with increasing perchlorate dose. The most sensitive life stages were shown to be the pregnant and postnatal female (LOEL of 0.1 mg/kg b.w. per day). The authors compared this to the human study of Greer (2002), where individuals given perchlorate at 0.4 mg/kg b.w. per day for 14 days did not have serum TSH levels that were significantly different from controls, and concluded that therefore the rat must be more sensitive than humans after 14 days exposures. However, no comparison was made with pregnant or postnatal humans exposed to perchlorate for the same period.

Similarly, for T3 levels, data from a range of studies was compiled and results showed pronounced dose-response effects on rats with a LOAEL in pregnant rats, postnatal dams and neonates of 1 mg/kg b.w. per day for 14 days. The LOAEL for fetal rats and non-pregnant adult rats was 0.01 mg/kg b.w. per day. This was compared with data in humans (Greer et al, 2002) and Lawrence et al. (2000, 2001) who did not see effects with doses as high as 0.48 mg/kg b.w. per day for a 14 day period. However, these studies on humans do not include data on pregnant women or newborns, so comparisons were not made for these subpopulations.

Finally, for longer term exposures, comparisons were made for a number of species. The results are summarised in the following table published by Lewandowski et al. (2004):

Lowest Observed Adverse Effect Levels (LOELs) in mg/kg-day for the effect of perchlorate on thyroid hormones as reported in various studies

Thyroid hormone measurement	Adult rat	Pregnant rat	Fetal rat	Neonatal rat	Postnatal rat dam	Female mouse	Pregnant rabbit	Adult human
TSH								
Subacute (14–38 days)	0.1	**0.01**	0.1	0.1	**0.01**	2	None (100)	None (0.5)
Subchronic (90 days)	0.2	na	na	na	na	**0.06**		None (0.48)
T_3								
Subacute (14–38 days)	**0.01**	1.0	**0.01**	1.0	1.0	None (30)	None (100)	None (0.5)
Subchronic (90 days)	**0.01**	na	na	na	na	None (30)		None (0.48)
T_4								
Subacute (14–38 days)	3	**0.01**	1.0	1.0	0.1	0.2	30	None (0.5)
Subchronic (90 days)	**0.01**	na	na	na	na	1.0		None (0.48)

Note. The lowest value reported in all studies addressing a particular species/lifestage is shown. The lowest LOEL value for each hormone measurement is shown in bold text. Sub-chronic human data are from occupational studies which are assumed to involve at least 90 days of exposure. na—Not applicable. This life stage is shorter than the duration of a chronic exposure. none—No LOEL reported. No effect was observed at the highest dose tested, which is identified in parentheses.

Figure 1 –Obtained from Lewandowski et al., 2004

While the data clearly shows a higher sensitivity for acute effects for the adult rat in relation to all the other species, including adult humans, again, no comparison was

performed in relation to more vulnerable subpopulations, such as pregnant human, postnatal human or infant.

As the evidence above shows, lower doses of perchlorate are sufficient to affect iodide uptake, thyroid hormone and TSH levels in rats, compared to humans. A number of physiological differences between both species have been highlighted as likely contributors to the toxicological differences. These are:

- **The half-life of thyroid hormones in adult rats is significantly shorter than in humans.** As a consequence, the rat is significantly less able to compensate for perturbations in its thyroid axis than humans following exposure to thyroid disrupting chemicals (Lewandowski et al., 2004; Fisher et al., 2012).

- **The duration of exposure to perchlorate that is required to cause disturbances in circulating levels of serum thyroid hormones is shorter in the rat compared to humans.** This is considered to be due to the rat thyroid gland having a smaller store of iodinated thyroglobulin (colloid), compared to humans, that is quickly depleted when iodine is limited (ASTDR, 2008). Close to birth, human fetal stores of iodine are also much greater compared to rat fetal levels, and the weight of the thyroid gland (relative to body weight) is also lower in the rat fetus, indicating the immature nature of the thyroid gland in the rat fetus near birth (Fisher et al., 2012). Nevertheless, it is important to point out that the newborn human has no stored thyroid hormone, and given that the ½ life of its T4 is much shorter than in adults, its requirements of iodine are high, in comparison with adults.

- **The occurrence of thyroid tumours (papillary/follicular adenomas and carcinomas), following exposure to perchlorate, have been noted in rats and mice.** There has been no direct evidence of perchlorate causing thyroid cancer in humans (ATSDR, 2008). The development of thyroid tumours following exposure to perchlorate is seen as unlikely in humans (NRC, 2005).

The implications of these physiological differences between humans and the rat led to the argument that 'the rat is a problematic model to use in estimating human health risks for chemicals that perturb thyroid function' (Lewandowski et al., 2004). The examination of T3 and T4 and, particularly, TSH responses to perchlorate in rats in comparison with humans has led to the suggestion that humans have a greater potential for adaptation after exposure to chemicals, without suffering changes in levels of serum thyroid hormones. Consequently, this evidence seems to suggest that the rat has much higher sensitivity to the effects of chemicals that affect the pituitary-hypothalamic-thyroid axis. This has led to the idea that whilst the rat can be considered a good model for screening thyroid disrupting chemicals and hazard identification, the current extrapolation tools routinely used in risk assessment are inadequate for extrapolating thyroid mediated disturbances and toxicity in rats to humans (Pickford, 2010; Lewandowski et al., 2004; Fisher et al., 2012).

In the **fipronil case study** we compile more recent evidence of the prevalence of transport proteins in the rat and in particular about TTR that should be taken into account when considering the usefulness of the rat for purposes of hazard identification and hazard assessment.

In any case, it is important to note that, due to a lack of robust epidemiological data, comparisons between the rat model and more sensitive humans subpopulations, such as pregnant women, infants, children, people with low iodine intake or pre-existing thyroid dysfunction and people exposed to other iodide-uptake inhibitors (such as thiocyanate in cigarette smoke) have not been conducted in detail. It is possible that, in these special cases, the high sensitivity of the rat model might be useful and relevant to estimate disturbances of thyroid function in humans. We believe this topic deserves further investigation in the future.

5.3.7 Ecotoxicology evidence

Based on the rat-human interspecies differences described above, it would be important to assess the applicability of alternative models for the testing of thyroid disrupting chemicals.

Due to the prevalence of perchlorate in the environment, several ecotoxicological test systems have been developed and optimised in order to evaluate the impact of chemicals able to disturb the thyroid on wildlife species.

5.3.7.1 Amphibians

Studies in various amphibian species have demonstrated the ability of perchlorate to inhibit thyroid-dependant metamorphosis (Reviewed in Pickford, 2010). Perchlorate was selected for the OECD Phase II optimisation and validation exercise for the Amphibian Metamorphosis assay.

Comparing perchlorate studies in rats and amphibians clearly indicates that both models are responsive to the impairment of thyroid hormone synthesis that results from the competitive inhibition of NIS by the perchlorate ion. Similar to the rat model, the most sensitive endpoint in the amphibian metamorphosis assay is the thyroid histopathology and the changes usually seen in thyroid structure are similar to those in the rat (e.g. increased gland size, colloid depletion and follicular hyperplasia). In terms of relative sensitivity of the rat and amphibian models, significant effects on thyroid structure have been reported at a dose level of 62.5 mg/kg day in the rat pubertal assay (Stoker et al., 2006) and a test concentration of 62.5 µg/L in the OECD Phase II validation of the amphibian metamorphosis assay (OECD, 2007), though in neither study was a NOEL achieved for this endpoint.

5.3.7.2 Fish

The pathways involved in human thyroid development, as well as tissue architecture, function and feedback regulation by the hypothalamus-pituitary-thyroid (HPT) axis are conserved in a variety of fish species making them promising models for the study of thyroid-active compounds. The responses of species such as fathead minnows, zebrafish and Eastern mosquitofish all have displayed thyroid follicular hypertrophy, hyperplasia and altered TSH in response to perchlorate (Jomaa et al., 2013; Raldúa and Babin, 2009, Crane et al., 2005; Schmidt et al., 2012; Mukhi and Patiño, 2007; Bradford et al., 2005). Similarly, a growing number of studies are using the stickleback as a model to study the impact of perchlorate on thyroid homeostasis and development (Bernhardt et al., 2011).

An assay to note here is the Zebrafish Eleutheroembryo Thyroid Assay (Thienpont et al., 2011). The endpoint measured in this assay is T4 concentration in thyroid follicles of 5 days postfertilization (dpf) zebrafish following 3 days of exposure to a water-borne chemical. On day 5 pf, larvae are fixed, and subjected to whole-mount immunofluorescence analysis using a polyclonal rabbit anti-T4 antibody. Total T4 immunofluorescence in follicular cells is then quantified and compared with untreated or vehicle-treated controls. The assay has been shown to respond significantly to perchlorate. To compare the sensitivity of rats and zebrafish eleutheroembryos to perchlorate, significant effects on thyroid gland histology were reported at a dose level of 62.5 mg/kg/day in a rat pubertal assay and 125 µg/L in the zebrafish Eleutheroembryo thyroid assay. However, it is difficult to compare dietary dose levels and aquatic test concentrations in the absence of robust data on uptake, distribution, and clearance of the test chemicals in the respective model organisms. According to a recent OECD scoping document, this assay has strong potential for identifying water soluble chemicals that directly affect thyroid function, and has the potential to be relevant to vertebrates in general if species differences in metabolism are taken into account.

5.3.8 References

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5.4 PBDE/PERFLUORINATED COMPOUNDS/PCB CASE STUDY

5.4.1 Summary

PBDEs induce clear neurodevelopmental effects in the human (cognitive effects, low IQ), but the picture that emerges in relation to thyroid disruption is far from clear. In some epidemiological studies, decreases in serum TH levels were seen, in others the opposite was observed. There are issues with data interpretation, due to concurrent exposures with other persistent pollutants also suspected of affecting thyroid hormone (TH) levels, the biological activity of unmeasured metabolites, the temporal variability in exposure and hormone levels measurements and gender-dimorphic responses that may be masked when separate analyses are not carried out for both genders.

In rodent studies, polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and poly- and perfluorinated alkyl substances (PFASs) reduce total and free serum T4, but without corresponding increases in TSH, a pattern described as "enigmatic". In order to shed light on the significance of altered circulatory TH levels, the effects of PBDES, PCBs and PFASs and their metabolites on the Hypothalamus-Pituitary-Thyroid axis, binding to proteins involved in peripheral TH transport, the regulation of intracellular levels of THs, binding affinity for thyroid receptors (TR), and downstream TH-mediated effects in some of the best studied organs (liver, brain, testes) were compared. The evidence available reveals both that these three classes of compounds can act, sometimes in opposite direction, centrally on the HPT axis, on tissue levels of TH by binding to peripheral transport proteins, cellular transporters or disrupting deiodinase action, and ultimately on TR-mediated genes. There is also clear evidence that one substance can elicit different responses in different tissues. It is therefore clear that interpretation of changes in peripheral TH levels is not 'one size fits all'. Similar patterns of effects peripherally may mask the complexity of concurrent disruption of TH action on several regulatory processes centrally, peripherally and in different tissues.

5.4.2 Scope and aims of this case study

The aspect of thyroid disruption discussed in this case study is the "enigmatic" pattern of effects of PBDEs on circulating thyroid hormone (TH) levels, namely decreases in TH that are not accompanied by the corresponding increases in TSH as expected from negative feedback. The emphasis was placed on comparing recent evidence related to downstream effects of PBDE exposure mediated via thyroid receptors (TRs) in different organs and tissues. This was compared with similar effects observed following exposure to polychlorinated biphenyls (PCBs) and per- and polyfluorinated alkyl substances (PFAS) that display the same enigmatic pattern. This case study should inform discussions related to the scientific significance of effects on circulatory TH levels and their implication for bioassay development, particularly:

- How should effects on circulating thyroid hormones T_3 and T_4 be interpreted in the absence of concurrent effects on TSH levels?
- What are the implications of tissue-specific responses for bioassay development?

5.4.3 PBDEs, PFAS and PCBs and their uses

Polybrominated diphenyl ethers (PBDEs) are persistent organic chemicals widely used as flame retardants in consumer products since the 1970s. The commercial mixture penta-BDE has been most often added to polyurethane foam used in furniture, while octa- and deca-BDE commercial mixtures have been used in electronics and other plastic products.

PFAS are a class of man-made organofluorine compounds widely used as surfactants and commonly differentiated by the length of their carbon chain. Perfluorooctanoic acid (PFOA) and perfluorooctanesulphonic acid (PFOS) have been the most extensively produced and studied. The strong C-F bonds exclude normal degradation pathways. Parent compounds are thought to be transformed to their acid, and both short- and long-chain perfluoroalkyl acids (PFAAs) are considered being metabolically inert (Danish EPA, 2015).

PCBs are organochlorine compounds formed of two benzene rings substituted by up to 10 chlorine atoms, resulting in up to 209 congeners. They were widely used as coolants and insulating fluids. In a manner akin to PBDE congeners, the lower circulating levels of T4 observed following exposure to PCBs has been assumed to be due to increased hepatic T4 clearance.

5.4.4 Observations from human studies with relevance to thyroid disruption

The epidemiological evidence of the neurobehavioural effects of PBDEs is now little disputed and will not be elaborated on here. Instead, this section will focus on the substantial number of epidemiological studies that have investigated the association between PBDE exposures and thyroid hormone levels.

In studies of **mixed-gender adult populations**, including specific ethnic groups such as Inuits (Dallaire et al. 2009) or occupationally exposed workers (Eguchi et al. 2015; Makey et al. 2016; Wang et al. 2010) as well as Chinese volunteers (Huang et al. 2014), no consistent pattern of effect emerges. Interestingly, Eguchi and colleagues (2015) reported a significant increase in TSH with total PBDE exposure when the analysis was carried for women only (67 women and 44 men were included in this study). Several studies have focused on **men**, including some highly exposed via their occupation (Julander et al. 2005; Pettersson et al. 2002) or recreational activities such as fishing (Bloom et al. 2008) and the consumption of fish (Hagmar et al. 2001; Turyk et al. 2008), whilst others were recruited via fertility clinics (Abdelouahab et al. 2011; Meeker et al. 2009; Johnson et al. 2013). Again, no consistent pattern of effect on TSH can be detected, however statistically significant increases in free T4 tended to be reported more often. The two Swedish studies examining small samples of workers in electronic waste dismantling plants carried out repeated measurements over time and noted considerable fluctuations in PBDE levels (Julander et al. 2005; Pettersson et al. 2002). One epidemiological study has considered **non-pregnant adult women alone**. Interestingly, as this study examined the association between PBDE exposure and clinical hypothyroidism rather than circulating TH levels, it included women currently taking thyroid medication, a population typically excluded from epidemiological studies in pregnant women (Oulhote et al. 2016). This cross-sectional study found a higher prevalence of hypothyroidism in highly exposed women, a significant relationship that became stronger when only younger women were considered.

No consistent pattern of effect is evident from studies in **children** (Gascon et al. 2011; Jacobson et al. 2016; Xu et al. 2014; Roze et al. 2009) or **adolescents** (Kicinski et al. 2012; Leijs et al. 2012). Both Jacobson et a. (2016) and Xu et al. (2014) reported that PBDE concentrations were higher in girls than boys, however no study has reported gender specific analysis of associations between TH and PBDE levels.

Due to the potential neurodevelopmental effects on the developing fetus, maternal thyroid hormone levels and PBDE exposure during **early and late pregnancy** have been the focus of considerable research efforts (Kim et al. 2013; Chevrier et al. 2010; Zheng et al. 2017; Abdelouahab et al. 2013; Vuong et al. 2015; Stapleton et al. 2011; Mazdai et al. 2003; Miranda et al. 2015; Lignell et al. 2016; Shy et al. 2012; Lin et al. 2011; Kim et al. 2011; Zota et al. 2011). Despite such efforts, no consistent associations between total PBDE exposure and maternal thyroid hormone levels have emerged, possibly because of the different stages of pregnancy studied. It should be recalled that TH and TSH levels during early pregnancy are exceedingly variable because of the enhanced requirements for TH particularly during early pregnancy and because human chorionic

gonadotrophin stimulates the thyroid in this period leading to a physiological decrease in TSH levels. Hence each lab has to establish norms for their thyroid measurements and cohort/ exposure studies require large numbers to obtain sufficient statistical analytical power.

It is nonetheless interesting to note that recently, whilst investigating Chinese pregnant women residing in the vicinity of an electronic waste recycling plant, Zheng et al (2017) found that a significant decrease in total T4 could be detected when restricting their analysis to women who had lived in the area for over 20 years. This suggests that the duration of exposure may be as important a variable as measured PBDE levels. Similarly, Zota et al (2011) did not find significant associations when considering the sum of PBDEs. However when they restricted their analysis to either hydroxylated PBDE metabolites or when they weighted PBDEs or their metabolite by their affinity for binding to transport proteins, significant TSH increases became apparent.

Correspondingly, the exposure of **newborns and infants** has also been the focus of considerable research (Lignell et al. 2016; Kim et al. 2009; Kim et al. 2011; Kim et al. 2012; Kim et al. 2015; Leonetti et al. 2016; Herbstman et al. 2008; Chevrier et al. 2011; Eggesbø et al. 2011). The majority of studies infer neonates' exposure from maternal blood, cord blood or breast milk and do not detect clear associations with newborns' TH levels. Two recent studies measured PBDEs in neonates' blood (Lignell et al. 2016) or placenta (Leonetti et al. 2016). Lignell et al (2016) report a positive association between total PBDE levels and total T3 at three weeks that was no longer significant at three months of age. Leonetti et al (2016) report a marginally significant association between total PBDEs and T3 in girl infants only, whilst in boys a marginally significant positive association with T3 sulfate was found. Placental PBDE congener levels tended to be higher for boys compared to girl infants.

Many of the studies cited here also investigated concurrent exposures with other persistent pollutants such as PCBs or PFASs. These co-exposures could confound any associations. Other confounding factors such as iodine status are not routinely corrected for, and methods to correct for blood lipids are subject to debate (Chevrier et al 2013). Furthermore, the majority of studies do not measure exposure to metabolites, particularly hydroxylated metabolites that are considered potentially more biologically active than the parent compound as will be illustrated in the following section. Additionally, it appears that PBDE levels are variable and that duration of exposure may be as important a variable as a concentration measured in one sample. Indeed, as TH regulate lipid metabolism and can also influence the metabolism of xenobiotics, some of the discrepancies in the literature observed with studies of associations with spot analyses of PBDEs adjusted for limits may be explained by reverse causality (Chevrier et al 2013). The temporal variability in both hormone levels and PBDEs may in any case hamper the detection of statistically significant associations. Further, gender dimorphic responses may be masked when studies have considered men and women, or boys and girls together. Finally, this summary has focused on reported associations with the sum of measured PBDEs and a detailed analysis of the consistency of significant associations with specific congeners was considered beyond the scope of this summary.

5.4.5 Observations from experimental studies – comparisons with PCBs and PFASs

Concerns arose regarding the PBDE congeners and their potential to disrupt thyroid processes due their structural similarity with T3. Reductions in serum levels of T4 are consistently observed in rodents following PBDE exposure. It has long been presumed that it is because PBDEs induce hepatic microsomal enzymes that increase T4 clearance, thereby reducing serum T4 in an analogous manner to the effects of phenobarbital (Bansal et al. 2014). However, the concurrent up-regulation in circulatory TSH expected from negative feedback mechanisms, as observed following anti-thyroid drug propylthiouracil (PTU) or phenobarbital-induced reductions in serum T4, is typically not detected. Similar patterns of effects have been observed with PCBs and PFAS (e.g. Zoeller et al., 2000; Lau et al., 2007).

The hepatic microsomal enzymes induced by PBDEs and PCBs are also involved in the detoxification of xenobiotic compounds and catalyse the addition of hydrophilic groups to the lipophilic phenolic structures resulting in metabolites that more readily excreted. These metabolites themselves may interfere more significantly with T3 effects on thyroid receptor (TR) activation than the parent compounds (Blanco et al. 2014). Moreover PBDEs can also displace T4 and bind antagonistically to TH transport proteins (Blanco et al. 2014; Bansal et al. 2014; Meerts et al. 2002).

In the following section, we briefly review the evidence on the binding affinities of PBDEs and their metabolites on TR isoforms, transport protein and their TH-mediated downstream effects in different organs.

5.4.5.1 The HPT axis

The production of Thyroid Releasing Hormone (TRH) in the hypothalamus stimulates the release of TSH by the pituitary which in turn initiates TH production in the thyroid gland. In a negative feedback loop, T3 levels regulate the production of TRH by the hypothalamus and TSH by the pituitary. A decrease in circulating THs would therefore be expected to increase the production of TRH and TSH. In rats, as in humans, the integrity of the HPT-axis is examined by administering synthetic TRH. Khan and Hansen (2003) evaluated the functionality of the HPT-axis in juvenile female rats by administering TRH following acute exposure to PCBs 95 or 101 (32 mg/kg for 2 consecutive days). Serum TSH levels were elevated in response to TRH but were only 40% of the response in controls. To our knowledge, no such TRH stimulation tests having been carried out following exposure to PBDEs or PFASs. Chang et al. (2008) investigated the secretion of TSH following TRH stimulation in the excised pituitary of rats previously dosed orally with 3mg/kg potassium PFOS per day for 7 consecutive days and saw no effect of PFOS exposure on TRH-mediated TSH release.

5.4.5.2 Pituitary

TSH is composed of two subunits synthesized separately. The GPHα subunit is identical to that of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), so it is the TSHβ subunit that determines the hormone's functional specificity (Lema et al. 2008).

In the fathead minnow, TSHβ mRNA was elevated in both sexes by the lower dose of BDE-47, consistent with negative feedback from decline in circulating T4. At the higher dose, transcripts for GPHα were depressed in both sexes without a change in TSHβ (Lema et al 2008), suggesting that alternative regulatory mechanisms or toxic effects may occur depending on the dose. Conversely, Bansal et al (2014) did not observe any increase in TSH mRNA in male rats exposed to DE-71.

The zebrafish has proven a popular experimental model to investigate the relationship between PBDE exposure and TSHβ gene expression (e.g. Chen et al. 2012; Zhao et al. 2016; Yu et al. 2010). Gene expression is however typically determined in whole body homogenates and this model does not allow the investigation of tissue specific responses. On the other hand, PFOS and PFOA exposure produced similar tissue specific responses in Atlantic salmon (Spachmo & Arukwe 2012). Both upregulated TSHβ gene expression in body region, and downregulated it in the head region.

Of ecological relevance, Couderc et al. (2016) measured PBDEs, PCBs and PFASs in the muscle tissue of the critically endangered yellow and silver European eels sampled in the Loire (France). Significant negative correlations between several PCB and PBDEs and lower plasma free T4 were detected in yellow individuals. It was not the case for PFASs. TSHβ gene expression in brain tissue was only significantly correlated with some PCBs.

5.4.5.3 Thyroid

Iodine is the major constituent of THs. Iodide is actively transported into thyroid follicular cells via the sodium-iodide symporter (NIS), where it is conjugated to thyroglobulin molecule (TG), a process catalysed by thyroid peroxidase (TPO).

FRTL-5 is a rat thyroid follicular cell line that has been used to study the influence of chemicals on iodide uptake and TH synthesis. Wu et al. (2016) found that BDE-47 was a non-competitive inhibitor of NIS (Ki = 77.8 μM). The authors also examined the transcriptional expression of three genes involved in TH synthesis, *Slc5a5*, *Tpo*, and *Tgo*, and three thyroid transcription factor genes, *Pax8*, *Foxe1*, and *Nkx2-1*, using rt- PCR in the same cell line. BDE-47 decreased the level of *Tpo* but no significant changes in the expression of any other genes were observed (Wu et al. 2016).

Intraperitoneal injection doses from 10 to 1000 μg/kg/day PCB118 for 13 weeks caused significant decreases in *NIS* and *TG* mRNA expression levels in rats (Tang et al. 2013). In pig thyrocyte cell culture, 306 nM PCB126 did not affect the gene expression of TG, TPO, or the TSH receptor (TSHR), while it downregulated the gene expression of NIS (Pocar et al. 2006). In FRTL-5 cells exposed to mono-ortho PCB 156 and its hydroxylated metabolites 4'-OH-PCB 159, 4'-OH-PCB 121, and 4'-OH-PCB 72, significant increases of TG concentrations were observed in the medium of cells exposed to 4'-OH-PCB 121 and 4'-OH-PCB 72 at concentrations as low as 1 nM, but not to the other compounds (Yang et al. 2008).

The activities of TPO, NIS, and TSHR mRNA in the thyroid of male rats were unaffected by 15.0 mg/L of PFOS in drinking water for 91 consecutive days (Yu et al. 2009).

In zebrafish, *Pax8, Nkx2-1, Slc5a5 and Tgo* were also upregulated following exposure to BDE-209, BDE-47 or DE-71 (Yu et al. 2010; Chen et al. 2012; Zhao et al. 2016). *Pax8*, and another gene involved in early thyroid development in the zebrafish, *hhex*, were also investigated following of exposure to PFOS. Du et al. (2013) found that both genes were upregulated when embryos were exposed to up to 500 μg/L PFOS for 7 consecutive days, while Shi et al. (2008) found that the effect was no longer significant at doses above to 1mg/L. No similar study was found for PCBs.

Effects related to iodide uptake were also investigated in clams (Song et al. 2016), minnows (Li et al. 2014) and gulls (Techer et al. 2016) following exposure to PBDEs, and chicken (Katarzynska et al. 2015) and gulls (Techer et al. 2016) following exposure to PCBs.

5.4.5.4 Cellular levels of thyroid hormones

Proteins in the serum reversibly bind THs establishing two pools of circulating T4 and T3 (i.e., protein-bound and free). The major circulating TH binding proteins differ in rodents and humans, with transthyretin (TTR) being the major protein in the rat and Thyroxine Binding Globulin (TBG) in humans. The free plasma TH is in equilibrium with tissues and affects TH signalling.

Peripheral transport - binding affinity for transport proteins

One hypothesis to explain the decrease in T4 often seen after PBDE exposure is that it is caused by the displacement of T4 from transport proteins.

The hydroxylated PBDE metabolites (OH-PBDE) have the potential to displace thyroid hormones from transport proteins, as they demonstrated much greater binding affinity to transport proteins than the hormones to both transthyretin (TTR) (Hamers et al. 2006; Meerts et al. 2000) and thyroxine-binding globulin (TBG) (Marchesini et al. 2008). Binding affinities of OH-PBDEs appear to be related to bromination, increasing significantly from 1 to 4 bromine atoms with no further increase with 5- and 6-brominated diphenyl ethers (Cao et al. 2010). In rodents, PBDE exposure has also been found to decrease Ttr mRNA expression (Szabo et al. 2008; Richardson, Staskal, Ross, Diliberto, DeVito & Birnbaum 2008). Binding to TTR and its mRNA expression following exposure to PBDEs have also been investigated in birds (Crump et al. 2008; Ucan-Marin

et al. 2009; Techer et al. 2016) and the zebrafish (Chen et al. 2010; Zheng et al. 2012; Chan & Chan 2012; Zhao et al. 2016).

Similarly, hydroxylated PCB metabolites (OH-PCBs) have a binding affinity for TTR similar to that of T4 and orders of magnitude greater than the parent compounds (Cheek et al. 1999; Hamers et al. 2011). The same cannot be said of their affinity for TBG for which they display an affinity *in vitro* about 100-fold lower than T4 (LANS et al. 1994). Recently, some lower-chlorinated OH-PCBs were identified as excellent substrates for cytosolic sulfotransferases (SULTs). These PCB sulfates were found to be able to bind TTR with an affinity similar to that observed for T4 (Grimm et al. 2013). The ability of OH-PCBs to displace T4 from TTR has also been observed in rodents, *in vivo* and *ex vivo* (Darnerud et al. 1996; Hallgren & Darnerud 2002). Binding to TTR and its mRNA expression following exposure to PCBs have also been investigated in birds (Ucan-Marin et al. 2009; Techer et al. 2016) and the frog (Shirey et al. 2006).

There still is relatively little information on the interaction of PFASs with TH transport proteins. PFAS tend to bind only weakly to TTR with binding potency 12.5-50-fold lower than the natural ligand T4. No binding activity could be detected for fluorotelomer alcohols (Ren et al. 2016; Weiss et al. 2009). PFAS do not appear to bind or display very low affinity for TBG (Ren et al. 2016; Weiss et al. 2009). The structure-binding analysis revealed that the binding affinities of PFASs to TTR are associated with the carbon chain length and the charge of the end group, with a medium chain length and a sulfonate acid group being optimal. In zebrafish, PFOS was found to down-regulate TTR gene expression in a concentration-dependent manner (Shi et al. 2009).

Transport into cells

THs transit in and out of cells via several different membrane transporters regulating intracellular thyroid hormone availability. Serum concentrations of circulating TH may therefore not be representative of intracellular T3 concentration, particularly if this transport system is disrupted. Solute carriers known to transport THs include monocarboxylate transporters (MCTs), Na+ /taurocholate co-transporting polypeptide (NTCP), organic anion transporters (OATs), amino acid transporters, and organic anion transporting polypeptides (OATPs). These carry both T4 and T3, and some, such as members of the MCT family facilitate not only cellular uptake, but also the efflux of THs. Transport is facilitated by specific substrate–transporter interactions and predominantly driven down concentration gradients. However, the physiological role of the transporters does not depend solely on their relative affinities for THs but also upon tissue- and cell-specific expression patterns. Finally, several thyroid hormone transporters do not transport thyroid hormones exclusively. For example, the OATPs transport a wide variety of both endobiotics and xenobiotics and possess multiple substrate binding sites (Bianco et al 2014). Evidence for PBDEs, PCBs or PFASs interactions with transporters appears sparse or particularly focused on the interactions of one class of substances with one class of receptors.

MCTs have been investigated with PBDEs in mice (Richardson et al., 2008) and more recently clams (Song et al. 2016), a rare study on molluscs. In juvenile mice, hepatic expression of Mct8 mRNA decreased significantly only at the highest dose of BDE-47 (100 mg/kg/day for 4 days), while in clams the same effect was observed in the µg/L range. No studies of PCBs or PFASs interactions on MCTs were found.

The interaction of three PFAS and **NTCPs** were investigated in human and rat hepatocytes and in a Chinese Hamster Ovary cell line (CHO) (Zhao et al. 2015). The results demonstrated that perfluorohexane sulfonate (PFHxS), perfluorobutane sulfonate (PFBS) and PFOS can be transported by both human and rat NTCP. This illustrates the point that the main concern of studies of PFAS and transporters is their long half-lives and the hepatic accumulation of PFOS in humans, rather than the impact of these interactions on intracellular TH levels.

Similarly, the cellular uptake of PBDEs and PFAs mediated by OATPs and OATs has been studied *in vitro* and *in vivo* and do demonstrate that these classes of compounds can

indeed interact with these transporters. However, some have considered the inhibition of cellular uptake of steroid hormone conjugates rather than THs (e.g. Pacyniak et al. 2011; Pacyniak et al. 2010; Yang et al. 2010; Yang et al. 2009). Of note, OAT4 is expressed in the human placenta. In an *ex vivo* study with human placenta, passage of PFOS and PFOA was modified by OAT4, indicating that OAT4 may decrease the fetal exposure to some PFASs (Kummu et al. 2015). In gulls, PBDE levels were positively correlated with transcription levels of OATP1C1 in the brain, while no such association was reported for PCBs (Techer et al. 2016).

Deiodinases

The modern paradigm of TH action recognizes that TH levels in individual tissues, and therefore TH signaling, can change even as serum hormone concentrations remain normal. The underlying mechanism is the local activation or inactivation of THs. The iodothyronine deiodinases types I, II, and III (D1, D2, and D3, respectively) regulate the activity of TH via removal of specific iodine moieties from the precursor molecule T4 (Bianco and Kim 2006).

Szabo et al (2009) observed decreased hepatic D1 enzyme activity and mRNA expression in male pups administered DE-71 perinatally. Inhibition of deiodinase activity in the human liver following exposure to some OH-PBDEs was confirmed *in vitro* (Butt et al. 2011). Kato et al (2004) also observed a significant decrease in the activity of hepatic D1 following treatment with PCBs in both Wistar and Gunn rats, a mutant strain of Wistar rats deficient in UGT1A isoforms, one of the UDP-glucuronyl transferase induced by PCBs (and PBDEs) and commonly assumed to be responsible for the decrease in serum T4. Treatment with PFOS similarly lowered hepatic DIO1 mRNA at 15.0 mg/L, however it was found to increase thyroidal DIO1 mRNA dose dependently (Yu et al. 2009). Dong et al. (2016) investigated the involvement of microRNAs (miRNAs) in PFOS-induced hepatotoxicity. They observed changes in transcripts that may mediate PFOS-induced effects on TH homeostasis including: activation of the CAR/PXR pathway, phase II/III enzymes, and deiodinase. These changes are consistent with low serum TH due to enhanced metabolic clearance of TH. However, most TH hepatic target genes were not altered in a manner consistent with reduced TH signalling.

The effects of exposure to PBDEs, PCBs and PFAS on deiodinase(s) have also been investigated in fish (Yu et al. 2010; Zhao et al. 2016; Dong et al. 2014; Buckman et al. 2007; Schnitzler et al. 2011; Thornton et al. 2016), birds (Techer et al. 2016), clams (Song et al. 2016), and frogs (Shirey et al. 2006).

5.4.5.5 TH mediated responses in cells and tissues

Binding affinities for TR

TRs act as ligand-activated transcription factors by inducing or repressing the transcription of genes containing thyroid response elements (TREs). There are three isoforms able to bind thyroid hormone, TRα1, TRβ1 predominately expressed in brain, liver and kidney and TRβ2 whose expression primarily limited to the hypothalamus, pituitary and sensory systems (ear and eye). In all vertebrates studied to date, distinct expression patterns of the TRα and TRβ isoforms suggest, and transcription studies confirm (Lezoualc'h et al., 1992), that TRs have tissue-specific and developmental-state specific functions (Lema et al. 2008).

PBDEs have been shown to interfere with the capacity of T3 to activate transcription through TR dependent mechanisms (Wang et al., 2011). However, using *in silico* modelling studies, some of their hydroxylated metabolites (OH-PBDEs) appear to exhibit higher binding affinity to TRs (Wang et al. 2011) suggesting PBDEs or their metabolites may be able to affect TH-regulated pathways in target tissues. The binding affinity and subsequent activity of different OH-PBDEs appear to be dependent on their degree of bromination. Low-brominated compounds appear to enhance T3 action whilst higher brominated compounds may function by interfering with T3 action (Ren et al. 2013).

Furthermore, the magnitude and/or nature of the effect on interference with T3 action at the cellular level may also depend on the TR isoform expressed in the target cell (Schriks et al. 2007).

PCBs and OH-PCBs have weak binding affinity for TRs but at least some OH-PCBs have been shown to be able to suppress TR-mediated transcription on several artificial TREs due to partial dissociation of TR from TRE (Cheek et al. 1999; Amano et al. 2010; Arulmozhiraja et al. 2005). Using combined *in vitro*, *in vivo*, and computational data, Ren et al (2015) found evidence to suggest that some PFCs also disrupt the normal activity of TR pathways by directly binding to TR.

T3-responsive genes contain TREs. In certain tissues and at certain developmental stages, transcripts for TRα and TRβ are auto-induced by T3. This auto-induction means that TR transcripts can be used as markers for assessing TH-induced activation of gene transcripts in target tissues (Lema et al. 2008).

Liver

The liver has long been considered as the major target organ for PBDEs, PCBs and PFAs. Enzymatic activity, due to their presumed role in T4 clearance, has been the focus of most of the research. In a study with male rat pups perinatally exposed to DE-71, Szabo et al. (2009) found that, in addition to glucuronidation, deiodination, active transport and sulfation may be involved in disrupting the levels of hormones available to bind nuclear receptors. While PCBs and PFASs have been also shown to affect deiodination, there are differences in their affinities to bind transport proteins, as previously discussed.

The liver has the second highest density of TRs in the body and several TH–responsive proteins have been identified. For example, as with fat tissue, TH stimulates lipogenesis in the liver by activating the expression of key enzymes involved in the synthesis of fatty acids, such as malic enzyme (ME) (Bianco et al 2014), that has been investigated following exposure to PBDEs, PCBs and PFAS.

Bansal et al (2014) investigated three TH-related genes in male rats and found an increase in *ME* expression instead of expected decrease. However, the two other TH-regulated genes *S14* and *Mdr1a* were not similarly affected, neither did they decrease. A possible explanation for this inconsistency may be that TH-related genes are also regulated by non-thyroidal pathways.

Hitomi et al. (1993) fed rats fed a PCB-containing diet for 1 day and observed elevated hepatic mRNA levels of ME prior to the induction of enzyme activity. The mRNA levels of ME in the kidney, lung, spleen, heart, and testis, were not affected by PCBs.

Female rats were given a single oral dose of 15 mg potassium PFOS/kg body weight. At intervals of 2, 6, and 24 h thereafter, measurements were made for serum fT4, TT4, TT3, reverse triiodothyronine (rT3), TSH, and PFOS concentrations, as well as liver PFOS concentrations, UDP-glucuronosyltransferase 1A (UGT1A) family mRNA transcripts, and ME mRNA transcripts and activity. ME mRNA transcripts were increased at 2 h and activity was increased at 24 h (Chang et al. 2008).

Hepatic cell cycle

In some cells T3 is a powerful inducer of cell proliferation. Among other mechanisms, T3 increases the levels of cyclin D1 leading to cell cycle transition from G1 to S phase. T3 also stimulates the activation of phosphorylated protein kinase Akt via PI3K signalling that in turns restrains the activity of GSK3β avoiding the exclusion of cyclin D1 from the nucleus and its proteasomal degradation (Blanco et al 2014).

In rodents, long-term exposure to PBDE leads to hepatocyte hypertrophy, necrosis and increased vacuolisation in rodents, which is suggestive of deregulation of the cell cycle (Blanco et al 2014). High perinatal PBDE exposure has been found to reduce protein levels of cyclin D1 and phosphorylated protein kinases Akt and GSK3β (Blanco et al

2014). The expression of TR isoforms was also decreased. It is mediated by the concentration of TH and a decrease in TH would be expected to result in an increase in predominant isoforms as an adaptive mechanism to maximise TH response, and vice-versa. Blanco et al (2014) suggested that this counter-intuitive result may be due to the direct activation of this autoregulatory mechanism by PBDEs and OH-PBDEs creating a state that mimic intracellular hyperthyroidism.

The decrease in cyclin D1 could be due to increased metabolism of TH or due to direct repression by increased availability of T3 (Lopez-Juarez et al., 2012). OH-PBDEs may also inhibit nongenomic actions of TH related to the activation of the PI3K/Akt pathway. An alternative explanation could be related to reactive oxygen species (ROS) synthesised as secondary products by several CYP enzymes and a decrease in the active form of Akt related to the increase in intracellular ROS levels (Blanco et al 2014).

Conversely, PCB-153 has been shown to induce hepatocytes proliferation and the mechanism invoked for this promoting activity was thought to be related to oxidative stress (Lu et al. 2003). In MCF10A epithelial breast cells, PCB-153 elicited similar effects to PBDEs, namely a decrease in cyclin D1 protein levels and inhibition of AKT and GSK3β phosphorylation (Venkatesha et al. 2010).

A low dose of PFOS (< 200 μM) stimulated the human liver cell line HL-7702 cell viability and increased cyclin D1 levels (Cui et al. 2015). PFOA treatment stimulated peripubertal mammary gland development in mice, but the increased cyclin D1 levels were attributed to a mechanism involving steroid hormones produced by the ovary (Zhao et al. 2010). On the other hand, PFOS was shown to attenuate the proliferation of neural stem cells C17.2 in a dose and time-dependent manner (X. Dong et al. 2016).

The section above demonstrates both that one compound can have different or even opposite effects in different cells and that substances with similar effects on circulatory levels of TH do not necessarily display the same properties in cells or tissues.

5.4.5.6 Brain and central nervous system

Because of the neurobehavioural effects observed in both experimental animals and in humans, the brain is fast emerging as potentially the most sensitive organ to toxic action by PBDEs. THs exert numerous important actions in the processes of neuronal maturation and differentiation and are involved in stem cell proliferation and neuronal differentiation in fish and mammals (Lema et al 2008, Lopez –Juarez et al. 2012). There could be direct PBDE effects on TR, but *in vitro* binding affinities are in the micromolar range, which seems high for this to offer a convincing explanation. On the other hand, studies examining expression patterns of TR-dependent genes showed activity in the nM range (Xiong et al. 2012; Ibhazehiebo et al. 2011).

Brain TRs

The TRα and TRβ play distinct roles in neural development. TRα regulates stem cell proliferation (Lopez-Juarez et al 2012) whereas TRβ mediates the differentiation of these newly proliferated cells into neurons and other neural cell types (Lema et al 2008, Bernal 2012).

In the fathead minnow, there are sex differences in the level of transcripts of TRα, while there are no differences between males and females in TRβ. Responses to BDE-47 exposure also demonstrated sex dimorphism for TRα (gene transcripts were elevated in females but not in males), whilst TRβ expression was depressed in both male and females (Lema et al 2008). These changes may result from the BDE-induced T4 decline or via interactions between BDE-47 and TRs or their corepressors. For example, PBDEs are known to interact with the nuclear steroid and xenobiotic receptor (SXR), that itself interacts with the corepressor SMRT (silencing mediator for retinoid and thyroid receptors). PBDE-induced impacts on SXR might therefore play a role in changes in TR gene transcription (Lema et al 2008). No changes in TR isoforms expression was

detected in the cortex and hippocampus of male rats following perinatal exposure to BDE-99 (Blanco et al. 2013), in contrast to what had been observed *in vitro* (Blanco et al. 2011).

RC3/Neurogranin

RC3/neurogranin is the rat homolog of the TH-responsive neuron-specific Neurogranin gene. Its mRNA expression has been studied in the fetal brain following PCB exposures. Maternal exposure to Arochlor 1254 increases RC3/Neurogranin mRNA expression in the fetal hippocampus, cerebellum and cortex of pups (Zoeller et al. 2000; Gauger et al. 2004; Lein et al. 2007). Interestingly, RC3/Neurogranin mRNA expression was depressed at the same life stage in an experimental hypothyroid rat model (perchlorate and methimazole treatment of pregnant dams), clearly demonstrating that PCB exposure does not simply mimic the effects of low TH (Bansal & Zoeller 2008).

Targeted literature searches did not identify any study of RC3/Neurogranin expression following PBDE exposure. For PFAS, it appears to have only been studied in birds. Some, but not all, PFAS increased RC3/Neurogranin mRNA expression *in ovo* (chicken embryo) or *in vitro* in chicken or herring gull embryonal neurons (Cassone et al. 2012; Vongphachan et al. 2011).

Brain-derived neurotrophic factor - BDNF

One of the key proteins regulated by TH is the brain-derived neurotrophic factor (BDNF), a neurotropin involved in the process of long-term potentiation (LTP), one of the best-known mechanisms underlying learning and memory. Reduction of LTP has been observed in rodent offspring following acute exposure to BDE-47 or chronic maternal exposure of BDE-209 (Blanco et al. 2013). The mRNA expression of BDNF is significantly downregulated in offspring hippocampi after perinatal exposure to BDE-99 and BDE-209 or co-exposed to BDE-47 and PFOS (Viberg et al. 2008; Blanco et al. 2013; F. Wang et al. 2011). However, BDE-47–induced changes in BDNF protein levels observed in neonates were rarely consistent with that of BDE-209 (Viberg et al., 2008, Wang et al., 2011).

Basic Transcription Element-Binding protein (BTEB)

The TH-regulated gene *BTEB* has been shown to mediate T3-induced neural differentiation and neurite branching via TH activation of TRβ. It encodes a zinc-finger transcription factor that binds GC-box domains to facilitate or inhibit TH-mediated gene transcription. The T3 induced BTEB protein also binds the promoter of the TRβ gene to regulate its autoexpression by THs (Bianco et al 2013).

Bianco et al (2013) found that female rats have lower levels of *BTEB* transcripts than males, but its expression was only reduced in males following perinatal exposure to BDE-99. PFOS exposure also resulted in up-regulation BTEB mRNA expression, however it presented an inverted U-shaped dose response pattern (Cheng et al. 2011).

Purkinje cells

Purkinje cells are large neurons located in the cerebellar cortex of the brain in vertebrates and play a role in motor movement. Perinatal hypothyroidism is associated with decreased dendrite arborisation and synaptogenesis of Purkinje cells and an *in vitro* experimental model using primary cell cultures of newborn rat cerebellum has been developed to study TH-induced dendrite arborisation. BDE-209 inhibited dendrite arborisation in this system whereas BDE-47 that does not suppress TR action did not supress Purkinje cell dendrite development (Ibhazehiebo et al. 2011). Some OH-PCBs exhibited the same suppressive properties on dendrite development in this system (Kimura-Kuroda et al. 2005).

Neuronal differentiation - Oligodendrocytes

Oligodendrocytes are myelin forming cells whose main functions are to provide support and insulation to axons in the central nervous system of some vertebrates. TH plays a role in oligodendrocyte differentiation and myelination during development or after injury. The effects of PCB exposure on white matter composition were examined by exposing pregnant rats to Arochlor 1254, with or without treatment with goitrogens as experimental hypothyroidism model. Both hypothyroidism and PCB exposure decreased the total cell density of the fetal corpus callosum and anterior commissure, but only hypothyroidism disproportionately affected oligodendrocyte density (Sharlin et al. 2006). An *in vitro* system using primary normal human neural progenitor (NHNP) cells. NHNP cells differentiate into neurons, astrocytes, and oligodendrocytes in culture and they express a variety of metabolism enzymes and nuclear receptors. Both T3 and PCB-118 treatments lead to a dose-dependent increase of oligodendrocytes was blocked by retinoic acid and the thyroid hormone receptor antagonist NH-3 (Fritsche et al. 2005).

5.4.5.7 Testis

The testis is also considered a thyroid-responsive organ and thyroid hormone plays a role in the development and function of Leydig cells (Sarkar et al. 2016). T3 is involved in induction of Leydig cell differentiation and steroidogenesis in rat testis. T3 stimulates mouse Leydig tumor cell (mLTC-1) steroidogenesis by upregulating the expression of StAR mRNA through SF-1. THs also control androgen biosynthesis and signaling through direct and indirect regulation of steroidogenic enzyme expression and activity. In rodent Leydig cells, T3 has been found to stimulate steroid production. Nonetheless, the mechanisms by which T3 regulates testicular steroidogenesis remain poorly understood (Sarkar et al 2016).

Mice treated with a high dose (950 mg/kg/day) of BDE-209 showed a marked down-regulation in testicular mRNA levels of SF-1, StAR, CYP11A1, 3β-HSD and 17β-HSD compared to controls (Sarkar et al., 2016). Moreover, immuno-labelling with PCNA, a nuclear proliferation marker, revealed that spermatogenesis was also decreased in the testis of treated mice suggesting arrest and suppression of spermatogenesis (Sarkar et al., 2016).

In adult Leydig cell cultures, BDE-47 and PBDE-710 increased the production of StAR protein levels or mRNA expression, respectively (Zhao et al. 2011; K.-L. Wang et al. 2011), while perfluorododecanoic acid inhibited StAR mRNA expression (Shi et al. 2010).

5.4.8 Discussion

In rodent studies, polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and poly- and perfluorinated alkyl substances (PFASs) reduce total and free serum T4, but without corresponding increases in TSH, a pattern described as "enigmatic".

To shed light on the significance of altered circulatory TH levels, the effects of PBDES, PCBs and PFASs and their metabolites on the HPT axis, binding to proteins involved in peripheral TH transport, the regulation of intracellular levels of THs, binding affinity for TRs, and downstream TH-mediated effects in some of the best studied organs (liver, brain, testes) were compared.

The evidence available reveals both that these three classes of compounds can act, sometimes in opposite direction, centrally on the HPT axis, on tissue levels of TH by binding to peripheral transport proteins, cellular transporters or disrupting deiodinase action, and ultimately on TR-mediated genes. There is also clear evidence that one substance can elicit different responses in different tissues. For example, the impacts of PBDE on gene transcripts do not conform to the expectations predicted by general hypothyroidism. TR-mediated downstream effects of PBDE exposure may be driven not only by circulating TH levels but also by the fact that PBDEs and their metabolites can

compete with T3 directly to either bind to transport proteins or modulate T3 interaction and activation of TR isoforms thereby deregulating the expression of TH-mediated gene expression. This overall picture is further complicated by the different binding affinities exhibited by specific PBDE congeners or corresponding metabolites (Ren et al. 2013), as well as their selectivity for different TR isoforms (Schriks et al. 2007). Furthermore, there is evidence that PBDEs can interfere selectively with T4 uptake into tissues (Bansal et al., 2014).

It is therefore clear that interpretation of changes in peripheral TH levels is not 'one size fits all'. Similar patterns of effects peripherally may mask the complexity of concurrent disruption of TH action on several regulatory processes centrally, peripherally and in different tissues. In short, serum T4 levels do not necessarily reflect downstream effects as PBDE, PCBs and PFAS can have multiple effects on hormone clearance, tissue uptake, receptor activation and regulation producing a complex pattern of effects on development and adult physiology.

The concept that circulating T3/T4 levels do not reflect tissue levels and tissue responses is now well accepted. Hence there is a need for both a complex suite of *in vitro* tests complemented with better endpoints for physiological effects of PBDEs and related chemicals,- particularly in the brain and specific brain areas.

5.4.9 References

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5.5 THYROID CANCER CASE STUDY

5.5.1 Summary

This case study considers the role of thyroid stimulating hormone (TSH) in the genesis of thyroid cancers, and compares and contrasts evidence from human studies, transgenic rodent models and traditional toxicological studies of thyroid carcinogenesis.

Two recent large meta-analyses of studies of the predictive value of TSH levels for thyroid cancers in humans have shown that elevated serum TSH levels are associated with increased risks of papillary and follicular thyroid cancer. However, these studies cannot resolve whether TSH plays a role in initiating thyroid cancer or whether it merely promotes the growth of pre-existing lesions.

Analyses of the role of TSH in normal follicular cells of the thyroid have revealed the hormone's unique character in stimulating cell proliferation and differentiation at the same time in the same cell. TSH is the decisive factor in follicular cell mitogenesis, but has to act in concert with other growth factors such as insulin and IGF1 which have a permissive role.

Improved understanding of signalling pathways in normal thyrocytes has proven invaluable in predicting key effectors that are dysregulated in human thyroid cancers. Genetic alterations found in human thyroid cancers involve the TSH pathway and constitutively activated elements of de-differentiating signalling pathways such as the RAS/RAF/ERK and PI3K/AKT systems. Accordingly, experiments with transgenic rodent models have demonstrated that TSH signalling is a necessary, but not sufficient, condition for thyroid carcinogenesis. In addition, the activation of de-differentiating pathways involving growth factor signalling is required for thyroid cancers to occur.

Chemicals that can induce elevated TSH levels in rodents are often also capable of producing thyroid tumours in rodents, mostly of the follicular thyroid cancer type. Modes of action that can lead to this disruption of the thyroid system include inhibition of iodine transport and thyroid hormone synthesis, increased thyroid hormone clearance through induction of hepatic conjugating enzymes and iodine deficient diets. The relevance of these effects for human cancer risk assessment is often debated in view of quantitative differences in the dynamics of the thyroid system of rats and humans. The higher turnover and clearance of thyroid hormones are thought to render the rat more sensitive to chemicals that produce thyroid hormone insufficiency and correspondingly elevated TSH with the risk of thyroid tumours. However, this does not explain why mice with their equally high thyroid hormone clearance are much less susceptible to thyroid cancer. Recent evidence from transcriptomics studies with conazoles that do not produce elevated serum TSH levels in rats suggest that signalling pathways leading to increased mitogenesis and de-differentiation might play a role in the formation of rat thyroid cancers, but further studies are necessary to substantiate these ideas. Until such evidence emerges there appears to be little reason to deviate from the USEPA and IARC guidance regarding the identification of thyroid carcinogens. This guidance rests on the presumption that rodent thyroid carcinogens may pose a cancer hazard to humans, and that in the absence of chemical-specific data humans and rats should be assumed to be equally sensitive.

5.5.2 Scope and aims of this case study

The role of thyroid disruption in the causation of human thyroid cancers is poorly defined. The toxicological literature describes many examples from rat models where chemically induced thyroid hormone (TH) insufficiency, with corresponding increases in thyroid stimulating hormone (TSH), has led to thyroid tumours. However, it is often argued that the rat is a poor model of thyroid carcinogenesis in the human. Purportedly less able than the human to compensate for perturbations of the thyroid hormone system, the rat is held to be particularly sensitive to thyroid disruption. For this reason, thyroid cancers in

the rat that arise via stimulation of the thyroid by TSH are often considered not relevant for human cancer risk assessment.

This case study examines the relationship between stimulation of the thyroid by TSH and the risk of developing thyroid tumours, first by considering evidence from human studies. In a second step, our understanding of the role of TSH in normal thyrocytes will be analysed in the context of knowledge about genetic alterations in human thyroid cancers and their impact on dysregulated signalling pathways. Against this background, thyroid specific transgenic rodent models will be assessed with the aim of elucidating what can be learnt about TSH signalling and thyroid cancer.

5.5.3 Human thyroid cancer

Thyroid cancers are the most rapidly rising type of all human malignancies in both women and men, with average annual increases of 6% year on year (Howlader et al. 2012, Kitahara and Sosa 2016). The reasons for this rise are not clear. Recognised risk factors include age, radiation exposure, obesity and a family history of thyroid cancer. Some argue that improved diagnosis might play a role, but the emerging consensus is that other factors are also involved.

There are several types of human thyroid cancers. The overwhelming majority of thyroid malignancies derive from follicular thyroid cells, with papillary thyroid carcinoma (PTC) representing around 80% and the more aggressive follicular thyroid carcinoma (FTC) 10-15% of all thyroid cancers. The remainder of follicular-derived thyroid neoplasms are poorly differentiated thyroid cancer and anaplastic thyroid cancer (Kirschner et al. 2016). A very small proportion of thyroid cancer derives from parafollicular C-cells (Xing 2013). PTC is the driver of the rapid rise in thyroid cancer in the USA (Kitahara and Sosa 2016).

Papillary thyroid cancer affects women more than men, with an incidence approximately 3 times higher in women than men. This is indicative of a significant estrogen-driven component in thyroid cancer in women. Accordingly, papillary thyroid cancer incidence peaks within a woman's reproductive period, while there is a linear increase with age among men (Wang et al. 2015).

5.5.3.1 Elevated levels of TSH as a predictor of thyroid cancer in humans

TSH stimulates the synthesis of TH, thyroid gland growth, induces changes in thyrocyte morphology, and regulates the activation of the sodium iodide symporter (NIS) and thyroid peroxidase (TPO), among other events.

Surprisingly, evidence for TSH as a predictor of thyroid malignancies in humans has emerged relatively late. Boelaert et al. (2006) were the first to describe that serum TSH is elevated in patients with malignant thyroid nodules when compared to subjects with benign thyroid tumours. Since then, several studies analysed in two large meta-analyses (McLeod et al. 2012, Zheng et al. 2016) have confirmed that higher serum TSH is associated with an increased risk of follicular and papillary thyroid cancer. The data presented by McLeod et al. (2012) predict a doubling of the odds of developing thyroid cancer for TSH serum levels between 0.65 and 4 mU/liter (Figure 1).

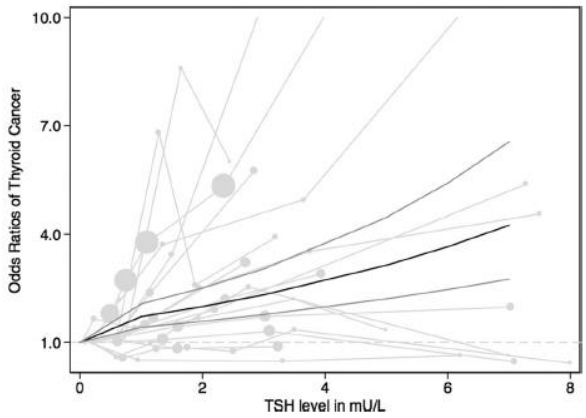

Figure 1, from McLeod et al. (2012): *Dose-response relationship for serum TSH and thyroid cancer odds ratios (OR). The black line represents the best estimate of OR, with confidence belts in dark grey. The grey dots and lines are from the underlying individual studies. The larger the dots, the smaller the variance in these studies.*

The studies that formed the basis of these meta-analyses were of a cross-sectional design. This limits the possibility of discerning whether raised TSH levels play a causative role in thyroid cancer. Without prospective studies it is difficult to decide whether TSH facilitates or even initiates thyroid cancer development, or whether it merely promotes the growth (and hence diagnosis) of pre-existing thyroid malignancies.

5.5.3.2 Evidence from studies of associations between iodine status and thyroid cancer

Recently, Zimmermann and Galetti (2015) have reviewed the role of iodine in thyroid cancer. The available evidence suggests that iodine deficiency is a risk factor for thyroid cancer, particularly the more aggressive FTC. However, the picture is confounded by other, as yet unrecognised factors that might also influence thyroid cancer. For example, thyroid cancer in the USA is steadily increasing since the mid 1980s, and iodine intake has declined over the same period. In contrast, Switzerland and China, both countries with iodine supplementation programmes and relatively stable iodine intake, have also experienced increases in thyroid cancer in recent years (Zimmermann and Galetti 2015).

To elucidate the role of TSH in thyroid cancer it is first necessary to establish the role of TSH signalling in normal thyrocytes. The ground is then prepared for a description of advances in our understanding of the molecular pathogenesis of human thyroid cancers and of genetic alterations in key signalling effectors.

5.5.4 TSH signalling in normal thyroid follicular cells

The role of TSH in thyroid cell proliferation and differentiation has been studied in *in vitro* models with established cell lines and in primary cell cultures. Permanent rat thyroid cell lines are the most popular model. Four species have been used mainly for primary cell cultures: the dog, pig, sheep and humans (Kimura et al. 2001).

Surprisingly, the signalling pathways and mechanisms that have been uncovered in these models differ in important aspects and depend strongly on the species, the culture conditions, the age (passage number) of the cells and even the "pre-history" of the cell cultures (Kimura et al. 2001, Roger et al. 2010). As a result, the interpretation of many of these studies is complicated. Generalisations from observations with *in vitro* models have to be made with careful consideration and usually require validation in *in vivo* systems such as transgenic rodent models or clinical observations in humans.

With these provisos, the features described below are based on observations with primary thyroid cell cultures from dogs which are viewed as most closely resembling TSH signalling in normal human thyroid cells (Kimura et al. 2001, Roger et al. 2010):

TSH is a glycoprotein hormone that interacts with the TSH receptor (TSHR), a G-protein coupled receptor. Upon binding of TSH, the receptor induces the coupling of various proteins which stimulate adenylate cyclase to form cyclic AMP (cAMP). This in turn activates the protein kinase PKA. In keeping with the main function of thyrocytes, this pathway also leads to the upregulation of components of the iodine machinery (including TPO and NIS), essential for the synthesis of thyroid hormones (TH).

Unlike many other mitogenic factors, TSH is capable of promoting cell division and differentiation programmes in the same cell at the same time. This is achieved in concert with other growth factors which signal via receptor tyrosine kinases, most importantly insulin and insulin-like growth factor (IGF1). TSH is the decisive mitotic trigger, while IGF1 is a permissive factor. The molecular basis for this relationship is in the activation of the cell cycle protein p27 by protein kinase A (PKA) via TSH and cAMP (Figure 2 a, from Roger et al. 2010).

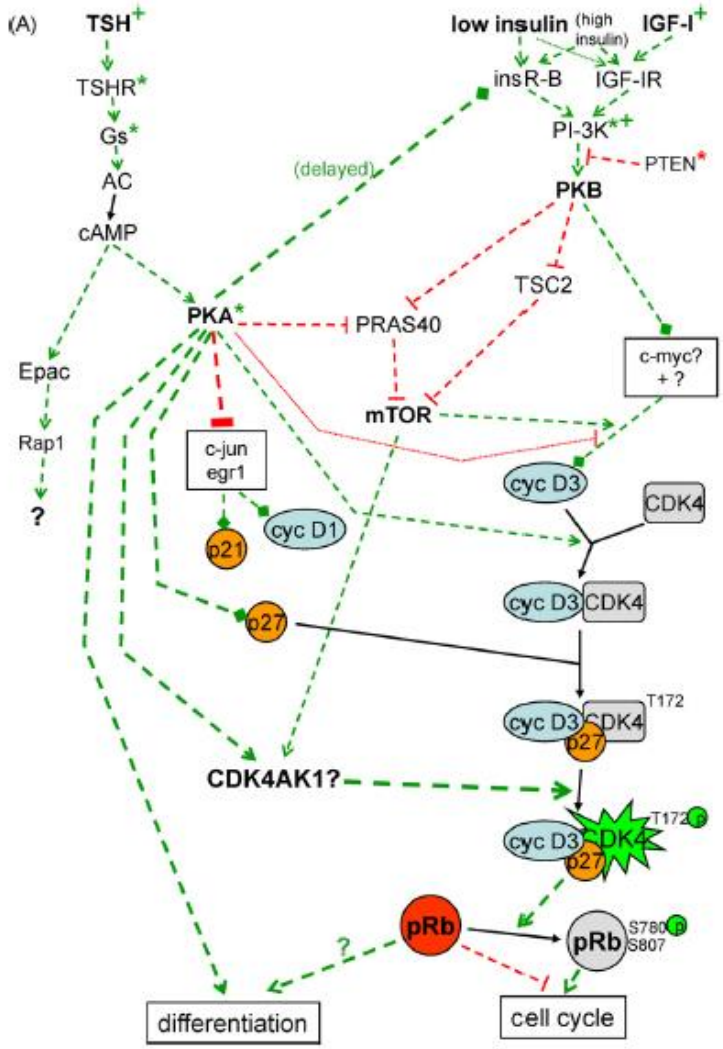

***Figure 2 A, from Roger et al. (2010)**: Summary of cooperative signalling of TSH with IGF1 derived from dog thyrocyte studies. Dashed green arrows are activations, dashed red lines are inhibitions.*

Protein p27 is needed to facilitate the assembly of the cyclin D3/CDK4 complex. This complex, a key element of the cell cycle machinery, phosphorylates specific residues on pRB, the "master brake" of the cell cycle. These phosphorylation events "release the brake" and promote cell cycle entry and differentiation signals at the same time. Ultimately, the function of IGF1 signalling is to provide sufficient levels of cyclin D3 for the aggregation of the cyclin D3/CDK4 complex. This is achieved by IGF1 signalling via PI3K and PKB. There are also negative feedback loops within thyrocytes, also activated

by TSH. These make sure that only one cell division ensues after stimulation of the cAMP/PKA/p27 pathway by TSH.

Accordingly, IGF1 on its own only weakly stimulates cell cycle entry: the limiting factor is p27 which has to be provided by TSH signalling via cAMP and PKA (Roger et al. 2010).

Other growth factors, such as epidermal growth factor (EGF) block the provision of p27, and instead signal via RAS/RAF/MEK/ERK to promote p21 and cyclin D1. These are needed for the assembly of another cell cycle complex, cyclin D1/CDK4. Cyclin D1/CDK4 phosphorylates pRB sites different from those phosphorylated by cyclin D3/CDK4. As a result, pRB now blocks differentiation, promotes de-differentiation signals similar to the epithelial-mesenchymal transition and strongly stimulates cell cycle entry (Kimura et al. 2001, Roger et al. 2010). These pathways appear to be required e.g. during the development of the thyroid (Figure 2 B, from Roger et al. 2010).

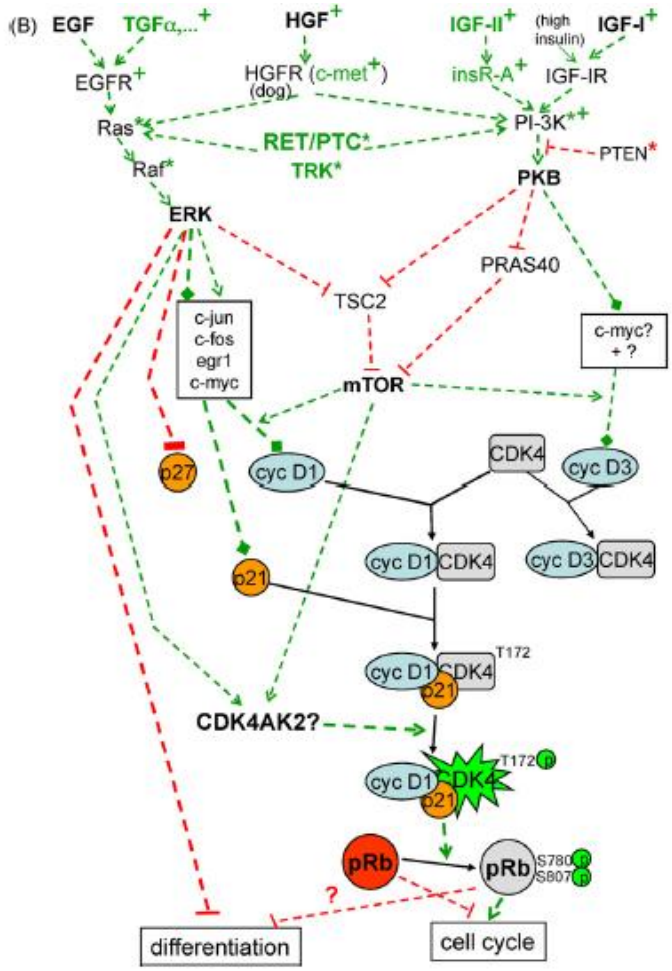

Figure 2 B, from Roger et al. (2010): *Summary of growth factor signalling in thyrocytes. Dashed green arrows are activations, dashed red lines are inhibitions. Effectors labelled in green are mutated in human thyroid cancers, see section below.*

Of note is a recent paper by Morgan and colleagues (Morgan et al.2016). When cell numbers were counted, instead of the usual measurement of DNA synthesis by incorporation of tritiated thymidine, no cell proliferation was observed in cultures of primary human thyrocytes exposed to TSH with or without IGF1. Although this paper casts doubt on some observations *in vitro*, the cell proliferative effects of TSH in cell cultures agree well with the hypoplasia seen in experimental animal after administration of agents that increase TSH levels (see below).

5.5.5 Molecular pathogenesis of human thyroid cancers and its recapitulation in rodent models

Human thyroid cancers and adenomas harbour genetic alterations that dysregulate elements of the normal thyroid cell signalling pathways, including components of the cAMP pathway and key effectors of MAP kinase pathways such as RAS/RAF/MEK/ERK. However, the origins and causes of these genetic alterations remain obscure.

5.5.5.1 Common genetic alterations in human thyroid cancers

Activating mutations of the TSH receptor that confer constitutive activity are found in thyroid adenomas. These mutations lead to continuous stimulation of the cAMP-mediated mitogenic pathways that involve PKA and p27.

The activating point mutation $BRAF^{T1799A}$ is the most frequently found genetic alteration in papillary thyroid cancer (PTC), the most prevalent form of human thyroid cancers. It encodes the constitutively active serin kinase $BRAF^{V600E}$ which continuously stimulates the MEK/ ERK signalling pathway and ensures that the cell cycle machinery receives constant activating signals. In addition, components of the iodine machinery (TPO, NIS) are down-regulated. The ensuing deficiency in thyroid hormones triggers a substantial rise in TSH (Xing 2013).

Activating mutations of all three *RAS* genes (*HRAS, KRAS, NRAS*) are prevalent in follicular thyroid cancers (FTC) and in PTC. These mutations de-regulate the MAP kinase and the PI3K - AKT pathways, with constant promotion of cell division.

Many thyroid cancers harbour gene amplifications and copy number gains in genes encoding elements of the PI3K-AKT pathway.

There are also gene translocations, the most frequent of which involves the receptor tyrosine kinase RET. Its intracellular kinase domain is fused with a variety of other gene fragments which possess dimerization domains. This brings the truncated RET kinase domains together to initiate autophosphorylation, leading to stimulation of RAS/RAF/ERK and PI3K/AKT with promotion of cell division (Xing 2013, Roger et al. 2010).

5.5.5.2 Transgenic rodent models of thyroid cancers

The relative importance of several of the genetic alterations found in human thyroid cancers has been studied in transgenic rodent models, typically involving mice. In these models, the genetic alterations under investigation were targeted to the thyroid. This was achieved by utilizing recombinase-mediated gene modifications in the Cre/Lox system (for reviews see Kim and Zhu 2009, Kirschner et al. 2016).

Here we focus on studies aimed at elucidating the role of TSH signalling in the development of thyroid tumours and at clarifying whether TSH may have a role in the initiation of thyroid cancer. These have all shown that growth stimulation by TSH is a necessary, but not a sufficient condition for cancer development. Concurrent activation of different MAP kinase pathways is also required for the occurrence of thyroid cancers.

Lu et al. (2009) employed mice with a knock-in of a dominantly negative, inactivating mutation of the thyroid hormone receptor beta gene (*THRB*), termed PV. The PV gene was derived from a patient with thyroid hormone resistance syndrome, the features of which are elevated TH and non-suppressible TSH. The PV mice recapitulated the human syndrome, with increased TH and TSH serum levels. As these mice aged, they developed follicular thyroid tumours (FTC). To investigate the importance of TSH signalling in this process, the effect of TSH signalling had to be eliminated in these mice. To achieve this, PV mice were crossed with TSH receptor knock-out mice. The offspring of these crosses showed impaired thyroid growth, but did not develop FTC. The experiment shows that growth stimulation by TSH is necessary for the development of FTC. However, TSH signalling alone is not sufficient for FTC to arise. Detailed analyses of PV mice uncovered constitutively activated MAP kinase pathways which were also required for FTC.

In a subsequent study (Zhao et al. 2012) the same group of researchers took this work further by utilizing a heterozygous PV mouse model with only 1 mutated PV allele. These mice do not develop FTC upon ageing. However, when TSH levels were raised by treatment with propylthiouracil, FTC appeared. The wild type siblings of the heterozygous PV mice did not develop FTC, despite being exposed to propylthiouracil. Further analyses of the FTC from the heterozygous PV mice showed that the cyclin D1 / CDK4 pathway was activated. To interpret this observation, it is important to realise that functional THRB suppresses cyclin D1. If that suppression of cyclin D1 / CDK4 signalling is lost, due to a dysfunctional THRB allele in the heterozygous PV mice, then FTC can develop under TSH stimulation via propylthiouracil. Again, this shows that activation of the TSH pathway is essential, but also demonstrates the importance of strong signalling to the cell cycle machinery for driving the development of FTC.

Franco et al. (2010) analysed the role of the most frequently found genetic alteration in papillary thyroid tumours, mutations of the *BRAF* gene. They used transgenic mice with *BRAF*^{T1799A} targeted to the thyroid. Cells expressing BRAF^{V600E} became transformed and progressed to PTC. Due to the accompanying downregulation of genes of the iodine machinery, these mice became severely hypothyroid with strongly elevated TSH levels. To examine the importance of TSH signalling in the process of PTC development, the BRAF^{V600E} mice were crossed with TSH receptor knock-out mice. Although BRAF^{V600E} was active in these crosses, the suppression of TSH signalling meant that PTC did not develop. Similar observations were made with BRAF^{V600E} mice that were crossed with mice with disrupted GSH signalling due to deleted G-protein genes. These findings show that the cooperation between TSH signalling and BRAF signalling in inducing PTC is mediated via cAMP and again demonstrates the importance of TSH in the formation of PTC.

Zou et al. (2015) studied the interactions between KRAS signalling and TSH in the development of FTC in mice. They utilized thyroid-specific knock-in mice with a constitutively active, mutated *KRAS* gene. In these mice, the RAS/RAF/MEK/ERK and PI3K/AKT pathways are activated, with stimulation of cell cycle entry and de-differentiating signals. As expected, there was mild thyroid enlargement in these mice, but no FTC development. However, when TSH levels were increased by treatment with propylthiouracil, massive thyroid enlargement occurred. After 14 months, these mice developed FTC.

Taken together, these observations underline the importance of TSH signalling in the development of thyroid malignancies. Without TSH signalling, malignancies do not develop. However, the data also show that growth factor signalling pathways that stimulate cell cycle entry and promote de-differentiating signals also need to be activated for thyroid cancer to occur. These results echo the epidemiological observations of associations between elevated serum TSH and thyroid cancer risk in humans (McLeod et al. 2012, Zheng et al. 2016).

5.5.5.3 The role of iodine in rodent models of thyroid cancer

The role of iodine in rodent models of thyroid cancer is of interest because of the rise in TSH that normally accompanies iodine deficiency. Accordingly, provision of iodine deficient diets to female rats for 6 to 20 months led to the appearance of follicular adenoma and FCT (Axelrad and Leblond 1955, Isler et al. 1958, Isler 1959, Schaller and Stevenson 1966, reviewed in Zimmermann and Galetti 2015). With iodine excess, there were increases in thyroid weight and histological changes, but no thyroid tumours were observed.

5.5.6 Toxicological studies of thyroid cancers in rats exposed to chemicals that produce TH insufficiency

As analysed by Hurley et al. (1998), 10% (24 of 240) of the pesticides screened by the USEPA until 1998 for carcinogenicity induced follicular thyroid cancers (FTC) in rodents. These malignancies appeared to be rat specific; the only pesticides that produced FCT in

the rat and the mouse were amitrole and ethylene thiourea. Furthermore, male rats appeared to be more sensitive to tumour induction than female rats.

With the possible exception of acetochlor, mutagenicity does not seem to play a significant role in the thyroid carcinogenicity of these pesticides. Instead, there were strong indications of thyroid disruption with 19 of the 24 pesticides considered by Hurley et al. (1998). Modes of action affecting the thyroid included: inhibition of iodine uptake (NIS), inhibition of TPO, inhibition of TH release, and enhanced TH clearance through induction of liver conjugating enzyme systems (UDPGT). All these modalities led to increases in serum TSH levels.

As is well established, persistently elevated TSH stimulates the thyroid to deplete its TH stores. Unable to keep up with the demand for TH, the thyroid follicular cells divide, leading to thyroid hyperplasia and eventually to cancer.

Thus, elevated TSH is regarded as the molecular initiating event that may lead to thyroid tumours in rodents (Dellarco et al. 2006). Of considerable current interest are agents that produce thyroid cancers in rodents via elevated TSH through the induction of conjugating liver enzymes (UDPGT) that remove TH. Examples of such chemicals are listed in Table 1 below.

Table 1: Chemicals that produce thyroid cancers through the induction of hepatic conjugating enzymes and elevated TSH

Compound	Effect	Species	Dose (mg/kg d)	Duration	Hepatic enzymes	Thyroid hormones	Comment	References
Fluopyram	FCA	Mouse	105	12 months	CAR+, PXR+	T4-, TSH+		Rouquie 2014
Nelfinavir mesylate	FCT, FCA	SD rat	300, 1000	24 months	UDPGT+, CYP 3A1+	TSH+		Burns-Nass 2005
Perchlorate	FCT	Wistar rat	1% in drinking water	18 months	na	na	silent BRAF mutations	Carmona-Lopez 2012
Thiazopyr	FCT, FCA	SD rat	44, 136	24 months	UDPGT+	TSH+		Naylor, McDonald 1992
Pronamide	FCT, FCA	CD BR rat	42	24 months	UDPGT+	T4-, TSH+		Bailey 1990; Papinemi 2015
Triadimefon	FCT	Rat	300, 1800 ppm food	24 months	UDPGT+	T3, T4-	TSH unchanged	Wolf 2006, Hurley 1998
Trifluralin	FCT	Fischer 344 rats	6500 ppm food	24 months	UDPGT+	T3, T4-, TSH+		Saghir 2008, Emmerson 1980
FCA: Follicular cell adenoma; FCT: Follicular cell tumour					Increases: +	Increases: +		
						Decreases: -		

Dellarco et al. (2006) considered the example of thiazopyr and discussed the applicability of this mode of action to human risk assessment within the 2006 IPCS Human Relevance Framework. They pointed out that although there are differences in the dynamics of the thyroid systems between the rat and the human, the fundamental mechanisms of regulation are similar. Thus, in principle, chemicals that induce TH decreases in the rat could also do so in the human, with consequent increases in TSH.

However, Dellarco et al. (2006) also emphasised quantitative differences between rat and human which in their opinion diminish the applicability of findings in the rat to human risk assessment: The increased clearance of TH in the rat (half-life of 12 h versus 5-9 days in the human) is compensated by higher TH production, with correspondingly higher basal TSH levels. As a result, so the argument, humans are less sensitive than rats to chemicals that produce TH decreases and corresponding TSH rises, with correspondingly lower thyroid cancer risks.

5.5.6.1 Unexplained species differences

However, as pointed out by Hurley et al. (1998), the discussion of dynamic differences in TH regulation between the rat and humans leaves one fact unexplained: Mouse and rat have similar TH hormone half-lifes, so both these species should be equally sensitive to TSH increases after exposure to agents that accelerate TH clearance. However, the mouse appears significantly less sensitive developing FCT when treated with agents that produce cancers in rats.

5.5.6.2 An alternative pathway leading to thyroid tumours in rodents, not involving elevated TSH serum levels

In general there is little information about gene alterations in chemically or otherwise induced rodent thyroid cancers that would allow comparison with the gene alterations

found in human tumours (see above). This gap has only recently begun to be filled with studies of conazole pesticides.

Wolf et al. (2006) have drawn attention to triadimefon (highlighted in red in Table 1) as a case that may help elucidating the events leading to thyroid tumours in rodents. Unlike many other chemicals which act via the hepatic route by induction of UDPGT and lead to TH decreases and corresponding TSH elevations, triadimefon is capable of inducing UDPGT and reducing TH levels, but does not lead to increases in TSH. Yet it produces follicular thyroid cancer in rats.

This unique effect pattern opens up the way for exploring alternative pathways in the induction of thyroid tumours in the rat, not solely dependent on elevated TSH.

Hester and Nesnow (2008) analysed this hypothesis further by conducting comparative transcriptomic studies in the thyroid of rats treated with triadimefon and myclobutanil. Both chemicals induce hepatic UDPGT to a similar extent with consequent decreases in TH. However, an increase in TSH was missing in both cases, yet only triadimefon, but not myclobutanil, produced thyroid tumours in a 24 month cancer bioassay.

After 30 days of treatment with triadimefon, there was expression of genes related to cell cycle G-S transition, the estrogen receptor alpha nuclear pathway, and platelet-derived growth factor signalling via MAPK. By matching core genes from the triadimefon gene set with those from human thyroid tumours, Hester and Nesnow (2008) found 10 common genes. This enabled them to produce a gene interaction network which they interpreted as one indicative of a cellular environment promoting cell proliferation. On the basis of these data, they hypothesised that triadimefon activates PPAR gamma with consequent transcription of genes controlling cell growth and proliferation.

In this, there are echoes with the studies involving transgenic rodent models which show the importance of growth factor-mediated signalling in the formation of thyroid cancers. It remains to be seen whether the hypothesis formulated by Hester and Nesnow (2008) is applicable to other chemicals that produce thyroid tumours via increased clearance of TH or by thyroid hormone imbalance induced by other mechanisms. To resolve this issue, classical toxicological studies of thyroid carcinogenesis will need to be complemented by transcriptomics analyses of the thyroid. If these de-differentiating and proliferative pathways can be shown to be operating in the rat thyroid also with other thyroid carcinogens, the relevance of observations in the rat for human risk assessments could be evaluated better.

5.5.7 USEPA and IARC guidance on rodent thyroid cancer

To support the interpretation of observations of thyroid tumours in rat studies, USEPA in 1998 developed science policy guidance (Hill et al. 1998). This guidance recognised that TSH plays a role in thyroid cancer and should be taken as an indicator of potential human cancer hazards, even if it turns out that the role of TSH is only permissive. On the other hand, this guidance recognises that the human may be less sensitive than the rat to developing thyroid cancer as a result of perturbations of the thyroid system. Against this background, USEPA adopted three science policy positions:

1. There is the presumption that chemicals that produce rodent thyroid cancers may pose a cancer hazard to humans.
2. In the absence of chemical-specific data, it is presumed that humans and rats are equally sensitive.
3. Thyroid gland enlargements following short-term changes in thyroid hormone levels are presumed to present a non-cancer hazard.

The International Agency for Research on Cancer (IARC) has used the following criteria for the application of mechanistic evidence in evaluations of rodent thyroid carcinogens which were developed by Capen et al. (1999) (IARC 2001):

Chemicals leading to the development of thyroid neoplasia through an adaptive physiological mechanism are viewed as belonging to a category different from those that produce thyroid neoplasia through genotoxic mechanisms or through mechanisms involving pathological responses with necrosis and repair. Agents that cause thyroid tumours in rodents through hormonal imbalance (without genotoxicity) can be distinguished from other thyroid carcinogens by a lack of genotoxic activity *in vivo* and *in vitro*. Hormone imbalance has to be demonstrated under the conditions of the carcinogenicity bioassay, and the mechanism through which the agent leads to hormone imbalance should be established. In cases where tumours are observed both in the thyroid and at other sites, they should be evaluated separately on the basis of the modes of action of the agent.

It should be assumed that chemicals capable of inducing thyroid follicular-cell tumours in rodents by interfering with thyroid hormone homeostasis can also interfere with thyroid hormone homeostasis in humans if exposure is at sufficiently high levels and for sufficient durations. At exposures not leading to alterations in thyroid hormone homeostasis, these chemicals are assumed not to be carcinogenic in humans.

Evidence for hormonal imbalance could include measurements of serum thyroid hormone and TSH and of morphological changes characteristic of increased TSH stimulation, including increased thyroid gland weight and diffuse follicular-cell hyperplasia and/or hypertrophy.

5.5.8 Conclusion

It would appear that traditional toxicological studies of thyroid carcinogenesis with their focus on analysing TH levels are likely missing key events leading to thyroid cancer in the rat. These events seem to revolve around the activation of de-differentiating and proliferative pathways in the thyroid, accessible only through functional and transcriptomics analyses not normally conducted in classical toxicological studies. Further studies of this kind are needed to substantiate the relevance of de-differentiating signalling pathways for the induction of follicular thyroid tumours in the rat. Until such evidence emerges there appears to be little reason to deviate from the USEPA and IARC guidance regarding the identification of thyroid carcinogens.

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6. ADDITIONAL BACKGROUND PAPERS FOR THE WORKSHOP

With the aim of keeping participants abreast of the latest developments in the area of thyroid disruption, a further set of background papers was prepared and circulated in advance of the workshop. These papers focussed on the following topics:

- Possible adverse effects in the human following disruption of the hypothalamic-pituitary-thyroid axis
- The thyroid system in fish, amphibians and invertebrates – a comparison with mammalian species
- Interpretation of data from epidemiology, experimental studies and wildlife data for the identification of thyroid disruptors
- Current OECD test methods for the identification of thyroid disruptors, their gaps and possibilities of improvements

6.1 BACKGROUND PAPER 1: POSSIBLE ADVERSE EFFECTS IN THE HUMAN FOLLOWING DISRUPTION OF THE HYPOTHALMIC-PITUITARY-THYROID AXIS

6.1.1 Summary

Thyroid dysfunction is characterised by under- or over-activity of the gland (hypo- or hyper-thyroidism, respectively). Hypothyroidism is more common than hyperthyroidism. Thyroid dysfunction has an impact on four major adverse health outcomes: neurodevelopment and brain function, cancer, cardiovascular disease and lipid metabolism, with impacts on body weight and body temperature.

Maternal subclinical hypothyroidism, characterised by normal thyroid stimulating hormone (TSH) with thyroid hormone (TH) levels towards the lower end of the population distribution, is associated with compromised cognitive development in their children (IQ loss). Recent studies also suggest associations with autism spectrum disorder, attention deficit hyperactivity disorder and schizophrenia.

Rises in the serum levels of TSH within the normal range are associated with increased risks of thyroid cancer, the fastest rising cancer among women and men.

Hyperthyroidism is associated with irregular heart beat and increased blood pressure. Hypothyroidism leads to a reduction in cardiac output, a decrease in heart rate and increases in peripheral vascular resistance. Hypothyroidism also leads to elevations in cholesterol levels.

Several studies have uncovered associations between exposure to pollutants and TH patterns typical of hypothyroidism. This is the case with combined exposures to perchlorate and thiocyanate, when accompanied by iodine deficiency. Similar associations have been shown with mercury, certain arsenic species, certain organochlorine pesticides, poly-aromatic hydrocarbons and perfluorinated compounds.

6.1.2 Introduction: the thyroid system

The focus of this paper is to give an overview of the adverse health effects in humans following disruption of the thyroid system. This will be addressed first by summarising the evidence from the clinical and epidemiological literature that describes associations of thyroid dysfunction with major diseases. The scene is then set to describe associations between thyroid disruption and exposure to chemical pollutants.

Thyroid hormones (TH) have essential roles in growth, development, especially of the nervous system, cell differentiation and homeostasis. TH are the ligands of several TH receptors, highly conserved ligand-dependent transcription factors present in all tissues,

but exhibiting different patterns of expression in different tissues, and which control gene expression by interaction with specific regulatory DNA sequences. There is also evidence of receptor-independent "non-genomic" response pathways which do not engage the binding to specific DNA sequences.

TH synthesis is controlled by the hypothalamo-pituitary axis. The production of Thyroid Releasing Hormone (TRH) in the hypothalamus stimulates the release of Thyroid Stimulating Hormone (TSH) by the pituitary which in turn initiates TH production (T3, T4) in the thyroid gland. Although T3 is the active TH – its affinity to thyroid hormone receptors is approximately 10 times higher than that of T4 – T4 is the prohormone that is most important in blood. Deiodination of the reservoir of T4 establishes an additional layer of regulation that ensures remarkable stability of circulating T3 levels. In a negative feedback loop, elevated T4 levels down-regulate the production of TRH by the hypothalamus and TSH by the pituitary. TH concentrations can also be modulated at numerous other physiological levels, including deiodination of T4 in peripheral tissues, removal of T3 by metabolism in the liver or interference with iodine uptake in the thyroid, which is required for hormone synthesis (Figure 1). For example, about 80% of T3 in the brain and pituitary are generated locally by deiodination (for more detail about the correlations between serum levels of various components of the thyroid system see Annex 1).

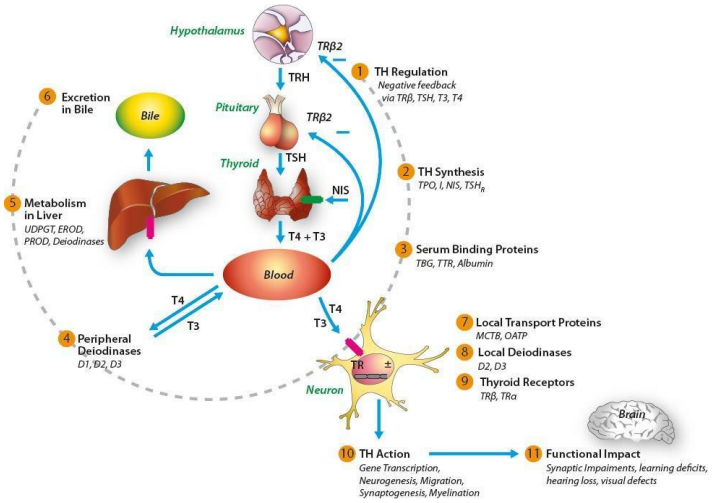

Figure 1: The thyroid system and its regulation (from: WHO/UNEP 2013)

Although there are some differences in detail, the basic principles of TH production, action and regulation are conserved across many taxa (see **Background Paper 2** The thyroid system in fish, amphibians and invertebrates – a comparison with mammalian species**, section 6.2)**.

6.1.2.1 Factors determining differential sensitivity to thyroid disruption across life stages

During the first trimester of pregnancy, maternal T4 is the only source of supply of TH to the fetus. The mother remains an important source of TH supply through to late gestation, contributing approximately 30% of the fetus' demand (reviewed by Ginsberg et al. 2007).

There are very low levels of TH in mother's milk. For this reason, newborns cannot anymore rely on mother's TH and have to synthesise their own supply. However, newborns have quite low functional reserves of TH, and the reserves available in the adult are not yet established. This makes newborns more vulnerable to perturbations of the thyroid system. Several factors play a role (Ginsberg et al. 2007):

- The serum half-life of T4 is lower in neonates than in adults (3 days vs 7-10 days).
- The rate of TH replacement in neonates must therefore be higher than in adults.
- The storage capacity of the neonatal thyroid is less than 1 day's worth of TH demand, compared to the adult gland which can store the demand of several months.

6.1.2.2 Diagnostic criteria: overt and subclinical hyper- and hypothyroidism

Overt hyperthyroidism is defined in terms of suppressed TSH serum levels and free TH levels above the reference range (see Jabbar et al. 2017 and reference cited therein). **Subclinical hyperthyroidism** is diagnosed when serum TSH is below 0.3-0.4 mU/L with free T3 and T4 levels within the population reference range.

Overt hypothyroidism occurs with TSH serum levels higher than 10 mU/L and T4 levels below the reference range. **Subclinical hypothyroidism** is diagnosed as "mild" with normal TSH serum levels between 4 and 10 mU/L and free T4 below the 10th percentile of the reference range. The criteria for "severe" subclinical hypothyroidism vary somewhat. It is sometimes defined as TSH levels exceeding 10 mU/L. Other authors classify "severe" hypothyroidism with TSH in the normal range and free T4 levels below the 5th percentile of the reference range (see the epidemiological studies on cognitive development in children discussed below).

As there is no consensus on the upper normal range of TSH, there is a controversy about the definition and clinical relevance of subclinical hypothyroidism (Hamilton et al. 2008).

6.1.2.3 Prevalence of hyper- and hypothyroidism

In general, hyperthyroidism is less common than hypothyroidism.

Overt hyperthyroidism occurs in 0.5% of the population (Cooper and Biondi 2012) and **subclinical hyperthyroidism** in 2% (Cappola et al. 2006).

Overt hypothyroidism is estimated to occur in 0.2-2% of the population (Tunbridge et al. 1977). **Subclinical hypothyroidism** is more common. Between 4 and 20% of the population show the symptoms, with women and the over 60 years old more likely to be affected (Biondi and Cooper 2008).

6.1.3 Adverse effects in the human resulting from thyroid disruption

Thyroid over- or underactivity is associated with four major diseases: Neurodevelopment and brain function, cancer, cardiovascular disease and alterations of lipid metabolism. More recent studies reveal effects also on the programming of other physiological effects during pregnancy.

6.1.3.1 Neurodevelopment and brain function

Of concern in relation to neurodevelopment and cognitive ability is hypothyroidism in the mother, due to the key role played by TH in neurodevelopment.

Maternal hypothyroidism and cognitive development in children

Several recent epidemiological studies have established the critical role of TH during the early stages of brain and cognitive development.

Of note is a study by Henrichs et al. (2010) in which the association between maternal thyroid function in early pregnancy and children's cognitive development was investigated. Henrichs and colleagues defined mild and severe hypothyroidism in pregnant women as free T4 levels below the 10th and 5th percentile, respectively, with normal TSH levels. Children's expressive vocabulary at 18 and 30 months of age was assessed. Both mild and severe maternal hypothyroidism was associated with delays in children's language development. Maternal TSH levels were unrelated to children's

language development. The study shows that maternal hypothyroidism is a risk factor for cognitive delay in early childhood.

Fan and Wu (2016) conducted a meta-analysis of six studies that investigated the relationship between hypothyroidism in women during pregnancy and its impact on neuropsychological development in their children. The outcomes assessed included intelligence scores and motor scores. This meta-analysis showed that children of women with subclinical hypothyroidism had mean intelligence scores several points lower than children from women with healthy thyroid status. The meta-analysis conducted by Wang et al. (2016) showed similar results.

Ghassabian et al. (2014) not only investigated the impact of maternal hypothyroidism on children's cognitive development, but also analysed brain morphology. The children of mothers with hypothyroidism in early pregnancy (defined as free T4 in the lowest 5% of the sample, with TSH in the normal range) scored 4.3 IQ points lower than the children of mothers with normal thyroid status. However, no associations with global brain volumes, cortical thickness, and brain surface area were found in these children.

The hippocampus is the brain component involved with short-term, long-term and spatial memory. Motivated by observations of abnormal hippocampus development in rodents with gestational TH deficiencies, Willoughby et al. (2014) investigated whether children of women who exhibited hypothyroidism during pregnancy also show hippocampal abnormalities. 54 children aged 9 to 12 years were studied, 30 from women with normal thyroid function during pregnancy and 24 from women with hypothyroidism. Children from mothers with hypothyroidism showed significantly smaller right and left hippocampal volumes and scored significantly lower on several memory indices, and these were correlated with hippocampal volumes.

Other neurological outcomes: autism spectrum disorder, attention deficit hyperactivity disorder, schizophrenia

Considering that transient gestational hypothyroxinemia in rodents induces cortical neuronal migration (a brain lesion resembling those found in autistic patients), Roman et al. (2013) investigated the association between maternal hypothyroidism in pregnancy (gestational weeks 6-18) and autistic symptoms in children. In line with earlier studies from this Dutch group, severe maternal subclinical hypothyroidism was defined as free T4 levels below the 5th percentile with normal TSH. Six year old children (4,039) were examined for behavioral and emotional symptoms suggestive of autism. Children of mothers with severe subclinical hypothyroidism had higher scores of autistic symptoms by age 6 years.

There is also evidence of associations with attention deficit hyperactivity disorder (ADHD). In a prospective, population-based Finnish birth cohort (9362 pregnancies; 9479 infants) Pakkila et al. (2014) analysed maternal TSH, free T4, and thyroid-peroxidase antibodies (TPO-Abs) from early pregnancy samples (5791 women). Teachers evaluated the children's ADHD symptoms at 8 years (5131 mother-child pairs). The authors analysed whether children with symptoms of lack of attention had mothers with hypothyroidism. The likelihood of inattention in girls, but not boys, increased with increases in maternal TSH during pregnancy. There were no associations with low maternal free T4 or the levels of antibodies against thyroid peroxidase. The authors concluded that increases in maternal TSH in early pregnancy showed weak but significant association with girls' ADHD symptoms.

Gyllenberg et al. (2016) tested the idea that maternal hypothyroidism from early to mid-pregnancy is associated with schizophrenia in their children. Maternal subclinical hypothyroxinemia (free T4 lower than the 10th percentile with normal TSH) was associated with an increased likelihood of schizophrenia.

6.1.3.2 Cancer

Thyroid cancers are the most rapidly rising type of all human malignancies in both women and men, with average annual increases of 6% year on year (Howlader et al. 2012, Kitahara and Sosa 2016). The reasons for this rise are not clear. Recognised risk factors include age, radiation exposure, obesity and a family history of thyroid cancer.

The overwhelming majority of thyroid malignancies derive from follicular thyroid cells, with papillary thyroid carcinoma (PTC) representing around 80% and the more aggressive follicular thyroid carcinoma (FTC) 10-15% of all thyroid cancers. PTC is the driver of the rapid rise in thyroid cancer in the USA (Kitahara and Sosa 2016).

Papillary thyroid cancers affect women more than men, with an incidence approximately 3 times higher in women than men. Accordingly, papillary thyroid cancer incidence peaks within a woman's reproductive period, while there is a linear increase with age among men (Wang et al. 2015).

Low levels of TH lead to rises in TSH, and TSH in turn stimulates thyroid gland growth. Boelaert et al. (2006) were the first to describe that serum TSH is elevated in patients with malignant thyroid nodules when compared to subjects with benign thyroid tumours. Since then, several studies analysed in two large meta-analyses (McLeod et al. 2012, Zheng et al. 2016) have confirmed that higher serum TSH is associated with an increased risk of follicular and papillary thyroid cancer. The data presented by McLeod et al. (2012) predict a doubling of the odds of developing thyroid cancer for TSH serum levels between 0.65 and 4 mU/liter, well within the normal range.

The studies that formed the basis of these meta-analyses were of a cross-sectional design. This limits the possibility of discerning whether raised TSH levels play a causative role in thyroid cancer. In any case, TSH has a clear role in thyroid cancer, whether in its initiation or in the promotion of pre-existing lesions.

Further details can be found in the **case study** on thyroid cancers (see section 5.5).

6.1.3.3 Cardiovascular disease

TH play an important role in the proper functioning of the heart and the cardiovascular system. TH regulate contractility and systolic function in heart muscle cells (cardiomyocytes). TH also influence heart rate and regularity of heart beat through effects on components of the adrenergic receptor complex and on various ion channels in cardiomyocytes. They help controlling the dilation of the vasculature.

The effects of TH on the cardiovascular system came to light at the end of the 19th century when a women with severe hypothyroidism was found to have an enlarged heart and thickened vessels (myxoedema) (see Jabbar et al. 2017).

Cardiovascular disease and overt and subclinical hyperthyroidism

The risk of atrial fibrillation and heart arrhythmia is increased in both overt and subclinical hyperthyroidism (Cooper and Biondi 2012). Patients with hyperthyroidism show an approximately 40% prevalence of pulmonary arterial hypertension (Jabbar et al. 2017 and references therein).

Studies of associations between subclinical hyperthyroidism and cardiovascular disease have produced more varied results, but a recent meta-analysis of relevant studies (Collet TH et al. 2012) has shown significant associations. In this study, individual data on 52 674 participants from 10 cohorts were pooled and coronary heart disease events and atrial fibrillation analysed. Subclinical hyperthyroidism was associated with increased total mortality, coronary heart disease and atrial fibrillation. Risks for coronary heart disease mortality and atrial fibrillation were higher for TSH levels below 0.10 mU/L when compared to subjects with levels between 0.10 and 0.44 mIU/L.

Cardiovascular disease and overt and subclinical hypothyroidism

Overt hypothyroidism affects the cardiovascular system in several different ways. It can lead to a reduction in cardiac output, a decrease in heart rate and increases in peripheral vascular resistance (Jabbar et al. 2017).

In subclinical hypothyroidism the most frequent abnormality is diastolic dysfunction where left ventricular filling is abnormal and accompanied by elevated filling pressures. Subclinical hypothyroidism can also impair the relaxation of vascular smooth muscles (Jabbar et al. 2017).

However, owing to differences in subjects' age, sex, TSH levels, or preexisting cardiovascular disease, observations of associations between subclinical hypothyroidism and cardiovascular disease in prospective cohort studies have varied somewhat. A recent meta-analysis of 55 287 participants from 11 cohorts (Rodondi et al. 2010) has addressed these issues by analysing the risk of coronary heart disease and total mortality for adults with subclinical hypothyroidism. The risk of coronary heart disease events and mortality increased with higher TSH serum levels, particularly in subjects with TSH levels above 10 mU/L. These risks did not significantly differ by age, sex, or preexisting cardiovascular disease.

Subclinical hypothyroidism does not seem to be linked with increased blood pressure (although some studies suggest such a link) (Jabbar et al. 2017).

Programming of metabolic set point and cardiovascular function during pregnancy

Rytter et al. (2016) investigated whether maternal hypothyroidism in week 30 of pregnancy may affect blood pressure and adiposity in their children when they have reached the age of 20 years. It was found that the children of subclinical hypothyroid women had higher systolic blood pressure and a tendency toward higher diastolic blood pressure when compared to the offspring of women with normal thyroid function. This association was not found in relation to measures of adiposity such as body mass index and waist circumference. Thus, maternal thyroid under-function during the third trimester of pregnancy may affect long-term blood pressure in their children.

Thrombosis and blood clotting

Overt and subclinical hyperthyroidism have been associated with alterations in the coagulation pathway (see the review by Jabbar et al. 2017), although it is too early to decide whether the observed biochemical abnormalities have any clinical consequences.

Alterations in coagulation parameters seen in subjects with subclinical hypothyroidism are thought to have a role in the development of atherosclerosis (Jabbar et al. 2017).

6.1.3.4 Lipid metabolism

TH regulate lipid metabolism through several mechanisms (reviewed in Jin and Teng 2014). TH contribute to the regulation of 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A Reductase (HMGCR), the rate-limiting enzyme in cholesterol synthesis (Mullur et al. 2014). In hypothyroid subjects, *HMGCR* mRNA levels are reduced.

TH also control the expression of genes involved in the hepatic cholesterol clearance, such as *LDL-R* and *ABCA1/ABGG5/8* (Jin and Teng 2014).

TH play a role in lipogenesis and lipolysis through the regulation of Lipoprotein Lipase (LPL) which is an essential enzyme responsible for removing triglycerides and lipoproteins (Kuusi et al. 1988, Nikkila and Kekki 1972, Abrams et al. 1981).

Hyperthyroidism and lipid metabolism

In patients with overt hyperthyroidism, the serum levels of total cholesterol are decreased, while triglyceride levels are either slightly elevated, normal or reduced (Tan et al. 1998, Abrams and Grundy 1981, Heimberg et al. 1985) and oxi-low density lipid levels are increased (Sundaram et al. 1997, Azizi et al. 2003). The reasons for this mild hypertriglyceridemia in patients with overt hyperthyroidism are not clear.

Hypothyroidism and lipid metabolism

Overt hypothyroidism is accompanied by increased levels of serum total cholesterol, low density lipids and lipoproteins, while serum levels of triglycerides and high density lipoprotein are normal or only slightly increased (Pearce 2012).

The results of studies of serum lipid levels in patients with subclinical hypothyroidism are rather varied (Pearce 2012). The lipid profile of patients with subclinical hypothyroidism with higher serum TSH level is similar to that in patients with overt hypothyroidism.

Other pregnancy outcomes

Su et al. (2011) investigated the effects of maternal thyroid dysfunction on pregnancy outcomes and infant development. In 1017 women with singleton pregnancies TH serum levels were analysed during the first 20 weeks of pregnancy. The outcomes investigated included fetal loss, malformations, birth weight, preterm delivery, fetal stress, neonatal death, and infant development. Maternal overt hypothyroidism was associated with increased fetal loss, low birth weight, and congenital circulation system malformations. Subclinical hypothyroidism was linked with increased fetal distress, preterm delivery, poor vision development, and neurodevelopmental delay. Low T4 levels were related to fetal distress, small for gestational age, and musculoskeletal malformations. Women with elevated T4 serum levels experienced more spontaneous abortions.

6.1.4 Evidence of the possible role of chemical exposures in thyroid disruption from epidemiological studies

Because of the number of key proteins in the thyroid endocrine system, chemicals may interfere with thyroid hormone action at many different molecular initiating events (MIEs) (see Figure 1). This includes inhibition of iodine uptake, inhibition of hormone production (thyroid peroxidase), displacement of TH from serum distributor proteins (thyroid binding globulin, albumin, transthyretin), with increased clearance by liver conjugating enzymes, induction of liver conjugating enzymes, inhibition of TH converting enzymes, interference with cellular uptake of T3 and/or T4, and potential interactions at the TH receptor level. Many, but not all of these perturbations will lead to changes in the serum levels of TH, with follow-on effects on TSH levels.

Because methods for measuring changes in serum levels of TH and TSH are readily available and in widespread clinical use, epidemiological studies that aimed at elucidating the possible role of chemical exposures in disrupting the thyroid system have mostly relied on these outcome measures. Very few studies have used other indicators of disruption of the thyroid hormone system such as the binding capacity of thyroid hormone binding globulin.

Key epidemiological findings of thyroid disruption are discussed in the **case studies** on fipronil, maconzeb, PBDE (and PCBs and PFOCs) and perchlorate, and will not be dealt with here (see section 5). In this paper, we summarise evidence of human thyroid disruption by substances or combinations of substances not already covered in the case studies.

6.1.4.1 Exposure to multiple inhibitors of iodine uptake, low iodine status

Not only perchlorate, but also thiocyanate and iodine deficiency can negatively impact iodide intake into the thyroid gland and diminish TH synthesis. Steinmaus et al. (2013)

investigated the combined effect of all three of these factors on TH levels in humans. By using data from the 2007-2008 National Health and Nutrition Examination Survey (NHANES), they categorised subjects into exposure groups based on their urinary perchlorate, iodine, and thiocyanate levels and compared mean serum T4 levels between groups. The individual effects of perchlorate, thiocyanate and low iodine were rather small. However, subjects with high perchlorate, high thiocyanate, and low iodine combined had T4 levels 12.9% lower than subjects exposed to perchlorate, low thiocyanate, and with adequate iodine supply. These data suggest that combined exposure to perchlorate, thiocyanate, and low iodine can reduce T4 levels.

Similar results were obtained in a study from Thailand by Charatcharoenwitthaya et al. (2014). As would be expected from exposure to a iodine uptake inhibitor, rising levels of perchlorate were positively associated with increased TSH and with decreased T4 in first-trimester pregnant women. Thiocyanate exposure was also positively associated with TSH in a subgroup of pregnant women with iodine deficiency.

Suh et al. (2014) also analysed data available from the US NHANES database to assess the combined effects of exposure to perchlorate, thiocyanate and nitrate on circulating TH. Increasing urinary nitrate was associated with decreases in serum T4 levels in non-pregnant women with low iodine supply in the NHANES 2001-2002 data base. The authors conclude that risk assessments for perchlorate should consider co-exposure to nitrate and thiocyanate.

6.1.4.2 Heavy metals

Chen et al. (2012) investigated the relationships between TH and the levels of Pb, Hg, and Cd in blood and Cd in urine by analysing a sample of adolescents and adults from the 2007-2008 NHANES survey. While there were no associations with Pb, rising blood Hg levels were associated with decreasing levels of serum TH in adults. The opposite pattern was observed with Cd where increasing urinary levels were accompanied by rising serum levels of TH.

The association of Hg with decreasing TH levels was also apparent in women of reproductive age, suggesting potential negative impacts on brain development. These findings are in agreement with a proposed mechanism for Hg-induced TH insufficiency where Hg accumulates in the thyroid and reduces iodide uptake at the sodium/iodide symporter by binding to iodide (Nishida et al. 1986). Hg also inhibits TH deiodinases in peripheral tissues, enzymes important in supplying active TH (Soldin et al. 2008; Tan et al. 2009).

A somewhat weaker association between Hg and decreased TH serum levels was observed in the study by Llop et al. (2015) who analysed 1407 pregnant women participating in the Spanish INMA birth cohort study. Hg levels were marginally significantly associated with total T3 but not with free T4 or TSH. The association between Hg and decreased T3 levels was stronger among mothers who consumed iodine supplements.

Using the data from the 2007-2010 NHANES survey, Jain (2016 a) analysed associations between various arsenic variables in urine and free and total T3 and T4, TSH and thyroglobulin. The various chemical arsenic species had different effects on the TH profiles. For iodine deficient men, dimethylarsinic acid was associated with decreasing total T4 levels and correspondingly rising TSH. Arsenic adjusted for arsenobetaine was correlated with declining total T4 levels in both iodine deficient and iodine replete women. In iodine replete women, TSH levels increased with total arsenic and arsenobetaine.

6.1.4.3 Organochlorines and poly-aromatic hydrocarbons (PAHs)

Jain (2014) took the data from the 2001-2002 NHANES survey to analyse the relationships between TH and TSH and exposures to oxychlordane, p,p'-DDE, trans-

nonachlor, and heptachlor epoxide. In 20–39 year old iodine deficient males as well as iodine replete males, TSH levels increased with increases in **trans-nonachlor exposure**. In iodine replete females rising trans-nonachlor levels were associated with declining total T4 levels. In 20–39 year old iodine deficient females, **oxychlordane** exposure was associated with rising TSH levels. In iodine replete females, Mexican Americans had higher TSH levels when the exposure to oxychlordane was medium than when the exposure was low. In iodine replete females rising oxychlordane led to declines in total T4. In non-Hispanic black people, **heptachlor epoxide** led to decreases in total T4. In iodine replete females above age 60 years, there was a positive association between TSH and heptachlor epoxide levels.

In a similar vein, Jain (2016 b) examined the 2007-2008 NHANES survey data for associations between TH and the following poly-aromatic hydrocarbons (PAH): 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyfluorene, 3-hydroxyfluorene, 9-hydroxyfluorene, 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene, and 1-hydroxypyrene. For females, increased levels of 2-hydroxynapthalene, 2-hydroxyphenanthrene, and 1-hydroxypyrene were associated with elevated levels of total T3. For males, increased levels of 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, and 9-hydroxypyrene were associated with decreased levels of free T4.

Julvez et al. (2011) measured PCBs, DDE and HCB in maternal pregnancy serum and milk from a population-based mother child cohort of 182 children. TH and TSH were measured in maternal and cord serum. Resin T3 uptake ratio (T3RU) was also assessed as an estimate of the amount of thyroxine-binding globulin sites unsaturated by T4. The T3RU is high in hyperthyroidism and low in hypothyroidism. There were consistent inverse and monotonic associations between PCB, DDE and HCB levels and T3RU after covariate adjustments. Associations with other thyroid parameters were not observed. T3RU was positively associated with improved performance on most of the neuropsychological tests. The authors concluded that these results suggest that PCB, DDE and HCB exposures may decrease the T3RU during early life, which is a proxy measure of the binding capacity of TBG.

6.1.4.4 PFOA and other perfluorinated compounds

Winquist and Steenland (2014) analysed 2109 cases of functional thyroid disease from an area surrounding the mid-Ohio River Valley where perfluorooctanoic acid (PFOA) was released from a chemical plant, exposing the surrounding community to PFOA for more than 50 years. Thyroid disease hazard ratios increased with rising exposure among women, suggesting associations for hyperthyroidism and hypothyroidism among women.

Webster et al. (2014) Associations between maternal serum PFASs, including perfluorohexanesulfonate (PFHxS), perfluorononanoate (PFNA), perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS) and repeated measures of maternal thyroid hormones, including free thyroxine (fT4), total thyroxine (TT4) and thyroid stimulating home (TSH) were examined. PFASs were not associated with free or total T4 or TSH among women with normal antibody titre against TPO. However, among the 9% of women with high TPO antibody, rises in PFASs were associated with increases in maternal TSH and with decreases in maternal free T4. The authors concluded that PFASs were positively associated with TSH, and weakly negatively associated with free T4 in the subset of pregnant women with high TPO antibody titre. PFASs may therefore exacerbate the already high TSH and low free T4 levels in these women during early pregnancy.

6.1.5 References

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6.2 BACKGROUND PAPER 2: THE THYROID SYSTEM IN FISH, AMPHIBIANS AND INVERTEBRATES – A COMPARISON WITH MAMMALIAN SPECIES

6.2.1 A comparison of thyroid systems in mammalian and non-mammalian species

In the last century, the thyroid system (as with many other hormones) was considered the preserve of vertebrates. However, more recently it has been shown that thyroid hormone (TH) functions also occur in invertebrate chordates, echinoderms (Heyland et al. 2004). Very recently, possible mollusc thyroid receptors have been identified (Kaur et al. 2015; Huang et al. 2015). Our understanding of the function of these more primitive thyroid systems is still in its infancy, therefore the focus of this document will be on non-mammalian vertebrate models. However, as our understanding grows, it may be necessary to also develop non-vertebrate thyroid test models.

Thyroid endocrinology is generally well conserved across vertebrate taxa, with similarities in thyroid synthesis, metabolism and mechanism of action. Therefore, chemicals which disrupt the thyroid system by targeting thyroid receptors as agonists or antagonist, thyroid hormone transport proteins, thyrotropin releasing hormone (TRH), or alter thyroid synthesis or metabolism could impact a wide range of vertebrate species.

6.2.1.1 Thyroid hormone synthesis and release

Iodine is the major constituent of THs. Iodide is actively transported into thyroid follicular cells via the sodium-iodide symporter (NIS), where it is conjugated to thyroglobulin molecule (Tg), a process catalysed by thyroid peroxidase (TPO). Tg had until recently only been investigated in mammals. However, Tg orthologs have now been identified in several non-mammalian vertebrates including xenopus, zebrafish, and sea lamprey (Holzer et al. 2016). NIS and TPO homologs have been identified in amphibians, fish and birds (Tindall et al. 2007; Opitz & Kloas 2010; Opitz et al. 2011; Katarzynska et al. 2015; Li et al. 2011; Opitz et al. 2006). Disruption to Tg, NIS or TPO would inhibit TH synthesis and therefore could be considered important targets for chemical disruption.

As in mammalian species, thyroid hormone (TH) release from thyroid follicles is regulated by pituitary thyroid stimulating hormone (TSH). Circulating TH negatively influences the activity of the hypothalamus and pituitary and, when TH levels are reduced or "disrupted," the activity of the hypothalamus–pituitary increases to elevate TH production by the thyroid follicles to restore its circulatory set point concentrations, and vice versa. Thus, the activity of the HPT axis is regulated by negative feedback (Carr & Patino 2011).

In mammals, thyrotropin-releasing hormone (TRH) controls the release of TSH. However, although present in non-mammalian vertebrates, it does not always act as a thyrotropin (TSH)-releasing factor, whereas corticotropin-releasing hormone (CRH) appears to be a potent stimulator of hypophyseal TSH secretion (De Groef et al. 2006). A recent review suggests additional close interactions between adrenal/interrenal and thyroidal axes, highlighting that corticoids also affect the expression of deiodinases (ID2) and thyroid hormone receptors (Watanabe et al. 2016).

6.2.1.2 Activating and de-activating deiodinases

In humans, 3 iodothyronine deiodinase (ID) enzymes are involved in converting, recycling and degrading T4 and T3. Homologs of ID genes have been found in non-mammalian vertebrates such as xenopus, zebrafish, chicken and alligator (e.g. NCBI gene search). Two of the deiodinases, ID1 and ID2, work in activating the outer ring-deiodinating pathway by converting T4 to T3 (considered the more active form of TH). In contrast, the inactivating or inner ring-deiodinating pathway is catalyzed primarily by ID3, which converts T4 and T3 to inactive metabolites (reverse triiodothyronine [rT3] and 3,3'-diiodothyronine [T2], respectively). Because of their essential function in controlling thyroid homeostasis, IDs have been suggested as potential biomarkers for thyroid disruption (Orozco & Valverde-R 2005). However, because of their central role in the HPT axis, IDs may also be prone to compensatory mechanisms, thus some chemicals may exert no effects on IDs although the thyroid system may be disrupted at other sites. Similarly, alterations observed in IDs may be driven by indirect processes (Jarque & Pina 2014). Jarque and Pina (Jarque & Pina 2014) warn that measurements of IDs alone could lead to erroneous conclusions and that changes in IDs need to be taken only as indications of interactions of chemical and thyroid system, with further evidence required to show physiological effects.

6.2.1.3 Thyroid hormone transporters and binding proteins

In the blood stream, thyroid hormones (T3, T4) are in the majority bound to transport proteins with only a very small percentage (~0.5%) being 'free'. In humans, around 75% of serum T4 is bound to thyroid binding globulin (TBG), 15% to transthyretin (TTR) and <5% to albumin. These three proteins are synthesized by the liver and secreted into the bloodstream, where they distribute thyroid hormones from the thyroid gland to cells throughout the body (Richardson et al. 2005). In humans, all three proteins have a higher affinity to T4 than T3. TBG has the highest affinity for T4 and T3 (1.0×10^{10} and 4.6×10^{8}/M, respectively), TTR has intermediate affinity (7.0×10^{5} and 1.4×10^{7}/M, respectively) and albumin has the lowest affinity (7.0×10^{5} and 1.0×10^{5}/M, respectively) (Alshehri et al. 2015).

Due to its higher affinity TBG had been considered 'more important' than the other transport proteins. However, due to the different dissociation rates TTR is actually

responsible for the majority of TH delivery to tissues. Alshehri *et al.* neatly use the metaphor of 'Goldilocks and the three bears' to describe the relationship, in that, TBG binds THs too tightly to deliver significant quantities under healthy conditions; albumin binds THs too loosely; and TTR has 'just right' dissociation rates (Alshehri et al. 2015). Similar dissociation rates for TTR have been found across species (Richardson 2007; Chang et al. 1999).

TBG is not found in all species, with albumin being found most frequently as the binding protein in a survey of 150 species of adult vertebrates (summarised in (Richardson 2002; Richardson et al. 2005)).

In many vertebrate species, the production of certain thyroid binding proteins is transient and related to specific developmental stages. For example, in rats TBG is only present during early development and senescence, while adult birds and many adult mammals have TTR as their main form of thyroid binding transport protein. In fish and amphibians, hepatic TTR synthesis is primarily found during early development, metamorphosis and smoltification (salmonids) (Richardson et al. 2005). Richardson et al. (Richardson et al. 2005) proposes that, across species, total TH levels are elevated in serum during critical developmental windows in part by regulated expression of genes coding for thyroid binding proteins with higher affinity to those already present in serum (Richardson et al. 2005). For example, TBG has a higher affinity for TH than TTR. More recently, sensitive molecular techniques have detected TTR expression in the liver of adult amphibians (Ishihara et al. 2012) and fish (Li et al. 2011), however, although these levels are still generally much lower than at metamorphic or developmental stages they likely still play an critical role in delivering TH to tissues .

Only one of the transport proteins, TTR, is synthesised in the brain of mammals (specifically choroid plexus) and involved in transporting TH across the blood-brain barrier. TTR is also synthesised in the choroid plexus of non-mammalian terrestrial vertebrates (birds, reptiles) but not aquatic vertebrates (fish, amphibians) (Schreiber 2002). TTR was first described in mammals as thyroxine (T4) binding protein. However, it seems mammals are the exception among vertebrates in respect to the function of TTR, as in teleost fish, amphibians, reptiles and birds TTR preferentially binds triiodothyronine (T3), which is the active form of thyroid hormone (TH) (Richardson 2015). It is also important to note, when comparing results in different test species, that total T4 and total T3 levels in blood vary between classes of vertebrates (Hulbert 2000).

Disruption to thyroid binding proteins (e.g. TTR), either by altered expression or by preferential binding, could be considered an important target for disruption in humans and other vertebrates.

6.2.1.4 Physiology

Although much of the machinery of the thyroid system is fairly-well conserved across taxa, there are some striking physiological differences. For example, teleost species can have heterotopic thyroid tissue, often with dispersed follicles (Figure 2), whereas, reptiles, birds and amphibians (Figure 2) generally have medial or paired glandular structures.

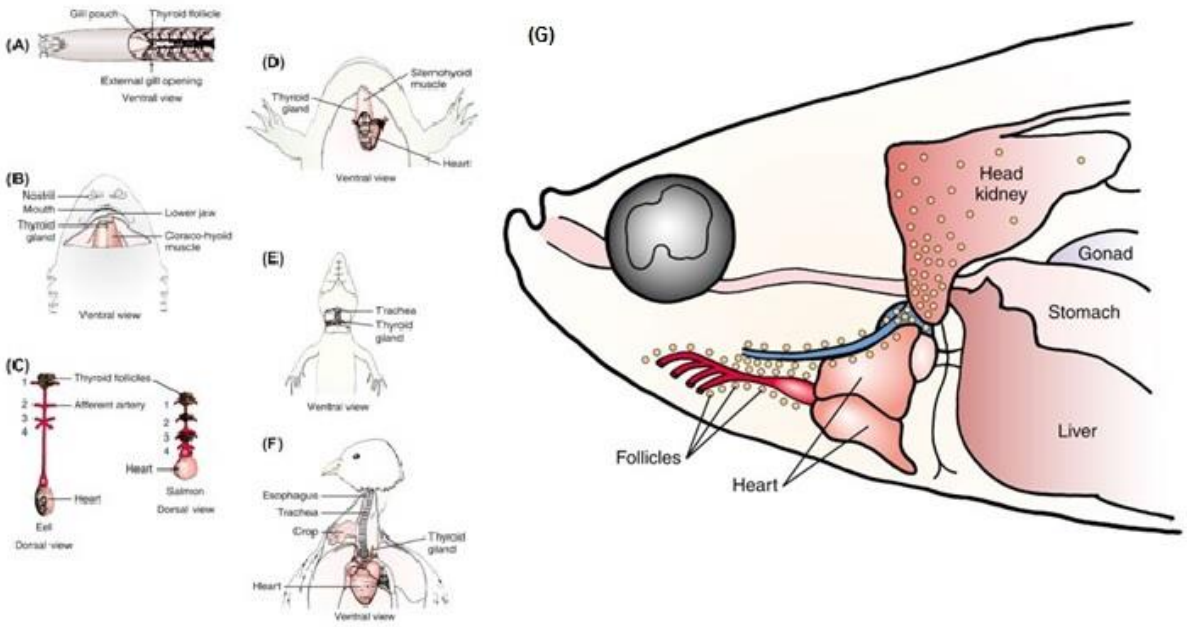

Figure 2. Location of thyroid tissue in non-mammalian vertebrates. (A) Scattered thyroid follicles in a hagfish (*Eptatretus burgeri*). (B) Discrete thyroid gland of the shark (*Triakis scyllium*). (C) Diffuse thyroids of the Japanese eel (*Anguilla japonica*) (left) and the Pacific salmon (*Oncorhynchus masou*) (right). (D) Paired thyroids in the bullfrog (*Rana catesbeiana*). (E) Medial thyroid gland in neck of the lizard (*Takydromas tachydromoides*). (F) Paired thyroid glands in a bird, the Japanese quail (*Coturnix coturnix japonicus*). (G) Scattered thyroid follicles (yellow dots) in Platy fish (*Xiphophorus maculatus*). Adapted from http://slideplayer.com/slide/686415/

6.2.2 Observations from experimental studies

6.2.2.1 Amphibian metamorphosis in validated OECD test guidelines

TH has a well established key role during early development in vertebrates. One of the most notable models for studying disruption to this function is amphibian metamorphosis (see Figure 3 D). However, many other vertebrates also require TH during key developmental events, such as hatching or post-hatch development in bird and reptiles (Figure 3 A-C) (De Groef et al. 2013), metamorphosis of flatfish (Schreiber et al. 2010), and parr-smolt transformation 'smoltification' in salmonids (Larsen et al. 2011).

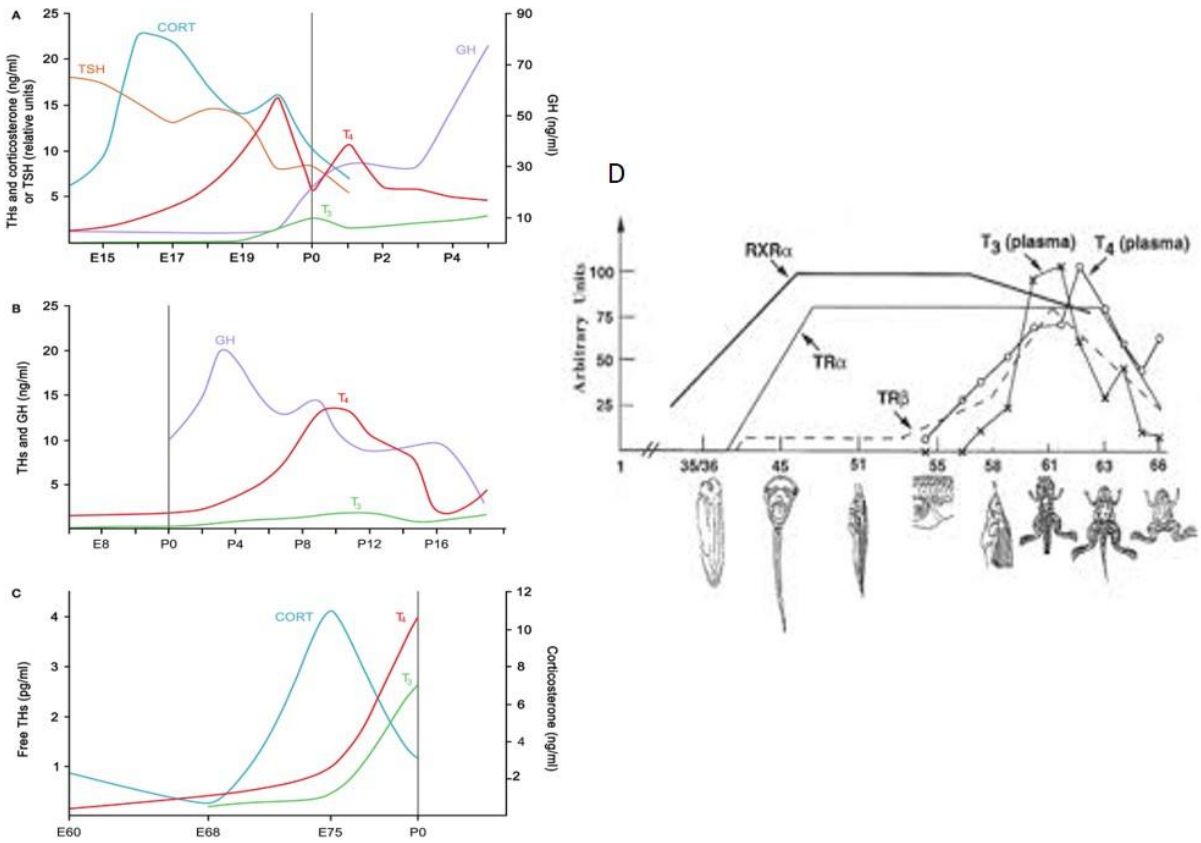

Figure 3 Generalized patterns of plasma hormones associated with hatching in (A) a precocial bird (chicken), (B) an altricial bird (European starling) and (C) a non-avian sauropsid (saltwater crocodile), E, embryonic day; P, post-hatch day; CORT, corticosterone; GH, growth hormone, (D) Correlation of the levels of endogenous TH and mRNAs of TRα, TRβ and RXRα genes with development. The embryos hatch around stage 35 and tadpole feeding begins at around stage 45. The plasma concentrations of T3, mRNA levels for TRα (solid line), TRβ (broken line) and RXRα (bold line) are based on published data are all plotted on arbitrary scales for ease of visualisation. Adapted from (De Groef et al. 2013; Wen & Shi 2016).

In 2010 Pickford conducted a critical comparison of mammalian and amphibian models for screening thyroid disrupting chemical. The most sensitive and robust endpoint in metamorphic xenopus assays (OECD screening test, TG 231) is generally considered to be disruption to thyroid histopathology (Pickford 2010).

6.2.2.2 Recent amphibian studies with additional endpoints

More recently several studies have integrated additional endpoints (e.g. molecular, TH measures) alongside traditional histopathological and developmental scores to help eluate the mechanism/target of disruption.

These studies have shown that modulations of gene expressions of components of the iodine machinery are often more sensitive effect markers. The same is true for genes related to TH metabolism and for gene expression in the brain.

Tietge *et al.* (Tietge et al. 2013) exposed Xenopus tadpoles to 2-mercaptobenzothiazole (MBT) a TPO inhibitor, for 7 or 21 days (Tietge et al. 2013). In both the 7 and 21-day studies thyroid histopathology was disrupted in all concentrations tested (≥ 18 µg/L), but effects were seen at a higher frequency with increased concentration and longer exposure. Metamorphic development was only significantly affected in the 21-day study. However, in the 7-day study, where additional endpoints (e.g. thyroidal T3, T4, NIS gene expression, serum TSH and T4) were measured, thyroidal T3 and T4 were found to be the most sensitive measures of disruption, whereas reduced serum T4 was a much less sensitive marker (Tietge et al. 2013).

Triadimefon (TDF) a triazole-derivative fungicide caused a reduction in developmental rates and significantly decreased whole body thyroid hormone (T4 and T3) levels in *X. laevis* tadpoles (Li et al. 2016). Downregulation of thyroglobulin (Tg) and upregulation of genes related to thyroid hormone metabolism (ugt1ab) in TDF exposed tadpoles may have been responsible for the decreased thyroid hormone concentrations. Treatment with TDF also significantly increased mRNA expression of genes involved in TSH possibly as a compensatory response to the lowered TH (Li et al. 2016).

In *X. laevis* tadpoles a high dose (5000 µg BDE-47/g food) of BDE-47 hindered growth and development; however, thyroid hormone-associated gene expression was downregulated in the brains of tadpoles at all doses tested (50, 500 or 5000 µg BDE-47/g food). These results show that BDE-47 disrupts thyroid hormone signalling at the molecular and whole-organism levels and suggest that gene expression in the brain is a more sensitive endpoint than metamorphosis (or gene expression in the tail). Furthermore, the altered gene expression patterns among BDE-47-exposed tadpoles provide insights into the mechanisms of PBDE-induced thyroid disruption (e.g. via reduced dio2 and organic anion transporting polypeptide 1C1 (OATP1C1) expression) and highlight the potential for PBDEs to act as neurodevelopmental toxicants (Yost et al. 2016). Histopathology of the thyroid gland was conducted as part of this study, but was inconclusive due to insufficient numbers for statistical analysis (n=4) (Yost et al. 2016).

6.2.2.3 Thyroid disruption in fish and birds

There are currently no specific OECD tests for thyroid disruption in fish or birds. Recent studies suggest several possible endpoints for thyroid disruption in fish and birds, as follows:

Thyroid histopathology

As with amphibians and rats, the histopathology of thyroid follicles of fish and birds can be assessed and has been shown to be sensitive to disruption. For example, exposure to perchlorate has resulted in disruption to thyroid follicles in stickleback fish (Furin et al. 2015), zebrafish (Mukhi & Patino 2007), Japanese quail (Chen et al. 2008) and northern bobwhite quail (Gentles et al. 2005).

TTR

In other models (*in vitro*, mammalian) PBDEs exhibit the potential to influence the levels of circulating THs by competitive binding of TTR (reviewed in (Yu et al. 2015)). In fish laboratory studies PBDEs (DE-71, BDE-209, or BDE-47) exposure resulted in reduced TTR transcriptional and protein levels and reduced whole body T4 concentrations (reviewed in (Yu et al. 2015)). Similarly, life-cycle exposure to BDE-47 resulted in significantly reduced (at 10 µg/L) T3, T4 plasma (F0 adult) and body (F1 eggs, F1 larvae) concentrations along with significantly reduced TTR expression in the liver of F0 adult (at 1, 5 and 10 µg/L) and F0 and F1 larvae (at 1 and 5 µg/L) (Zhao et al. 2016).

TTR expression was also significantly reduced in adult fathead minnow exposed to BDE-47 in the diet (41.22 µg/pair/day), however possible impacts on T3 or T4 were not measured in this study (Thornton et al. 2016). Yu *et al.* suggest that although the mechanisms of reduction in TTR levels are not well-understood, the down-regulation of TTR mRNA and protein levels caused by PBDEs exposure could impact the binding and transport of THs and thus pose a potential risk to thyroid functions (Yu et al. 2015).

In comparison to PBDEs, permethrin has recently been shown to up-regulate TTR and increased T3 and T4 concentrations in zebrafish larvae (Tu et al. 2016), *in silico* docking models indicated permethrin could also bind to TTR.

Deiodinase activity

Jarque and Pina (Jarque & Pina 2014) reviewed natural and chemical factors that can modulate deiodinases in teleosts. PBDEs, HBCD, PFOA, PFOS, pharmaceuticals and

metals have all been shown to modulate specific deiodinases levels in different tissues of fish (reviewed in (Jarque & Pina 2014)). However, as yet the exact mechanisms of disruption are not well characterised. For example, PBDEs (and metabolites) have been suggested to act directly on deiodinases expression, as 'T4 mimetic substrates' for deiodinases or indirectly due to pregnane X receptor (PXR) activation, where ID1 and ID2 are upregulated to try and maintain TH levels (reviewed in (Jarque & Pina 2014)).

Less information could be found for birds. However, in a chicken embryo model, TTR was down-regulated in the liver by *in ovo* exposure to hexachlorocyclopentadienyl-dibromocyclooctane (HCDBCO, brominated flame retardant) (Egloff et al. 2011). Whereas, 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) down regulated ID3 *in ovo* and *in vitro* (chicken embryonic hepatocytes) and decabromodiphenylethane (DBDPE) increased ID1 *in vitro* only (Egloff et al. 2011).
In a chicken thyroid explant experiment (*ex vivo*) PCB 126 and TCDD significantly increased expression of TPO and Tg, but not NIS, along with decreased T4 and T3 (Katarzynska et al. 2015).

Swim-bladder inflation

The involvement of TH in swim-bladder development in fish has been highlighted by Liu and Chan (Liu & Chan 2002). They demonstrated that in larval zebrafish excessive TH treatment (10, 40 nM T4, 5 nM T3) resulted in failure of the swim bladder to remain inflated. Co-treatment with amiodarone (TR antagonist, 50 nM) and methimazole (goitrogen, 0.3 nM) resulted in retarded swim-bladder development (but not individually), which could be 'rescued' by addition of 10 nM T4 (Liu & Chan 2002). The swim bladder consists of two chambers separated by a narrow duct. The posterior chamber is developed and inflated first, in what are described as 'swim-up' fry -this posterior chamber is inflated initially by the fish gulping air at the water surface. It operates as a vital hydrostatic organ to regulate buoyancy. The anterior chamber develops later and functions primarily as an acoustic resonator to aid hearing. TH is also important in regulating otolith ('ear' bone) (Schreiber et al. 2010) and lateral line (sensory organs) development/ regeneration in fish (Bouzaffour et al. 2010; Levin 2010).

PFOS has been shown to impair posterior swim-bladder inflation in developing zebrafish (Hagenaars et al. 2014). The swim-bladder impairment was seen alongside spinal curvature and resulted in decreased swimming ability (speed). However, no thyroid specific endpoints were assessed in this study. Similarly zebrafish developmentally exposed to 6-OH-BDE-47 show delayed swim bladder, fin, and pigmentation development (Macaulay et al. 2017). Impaired anterior swim-bladder inflation has been reported in zebrafish (350 µg/L MBT) (Stinckens et al. 2016) and fathead minnow (1000 µg/L MBT) (Nelson et al. 2016) exposed to thyroid peroxidase inhibitor 2-mercaptobenzothiazole (MBT). In these studies, thyroid endpoints were assessed. In fathead minnows MBT induced follicular cell hypertrophy (500 and 1000 µg/L), whole body T3 and T4 were reduced however, TPO mRNA suggested compensation (Nelson et al. 2016). In zebrafish T4 was reduced and anterior chamber surface was positively correlated with whole body T4 levels (Stinckens et al. 2016). The lack of effect on posterior swim-bladder inflation in these MBT studies was proposed to be related to the earlier development of the organ (compared to anterior swim-bladder) and maternal transfer of TH (Nelson et al. 2016; Stinckens et al. 2016).

Due to its vital importance in fish health, aquaculture papers also describe disruption to normal swim-bladder inflation. It has been linked to maternal nutrition in gilthead seabream (Tandler et al. 1995), suboptimal water temperature (Sanabria et al. 2009) in freshwater angelfish, and bathing eggs/fry in some aquaculture disinfectants i.e. hydrogen peroxide, acriflavin or methylene blue (Sanabria et al. 2009). Interestingly, the impacts of methylene blue on swim-bladder development were timing specific; exposure for up to 1 day post-hatch did not affect swim bladder non-inflation, but exposure from 2 days onwards significantly increased swim bladder non-inflation (Sanabria et al. 2009).

Behaviour

Exposure of zebrafish larvae to tetrabromobisphenol A (TBBPA) or propylthiouracil (PTU) resulted in quite similar visual and behavioural disruptions despite very different molecular responses of thyroid system-related genes (TRα, TRβ, TPO, TSH, DIO1, DIO2 and DIO3) (Baumann et al. 2016). Both TBBPA and PTU reduced relative eye size, morphology and pigmentation and altered ocular motor reflex. Swimming activity was also altered by both TBBPA and PTU, whereas light:dark preference was only altered in PTU treated fish (Baumann et al. 2016).

Altered behaviour has also been found in stickleback (fish) exposed to perchlorate (Bernhardt & von Hippel 2008). F1 fish showed a range of altered behaviours including those which might imped survival and successful reproduction i.e. nest building, spawning, nursery formation, or fry production (Bernhardt & von Hippel 2008).

Perchlorate has also been shown to impact bird behaviour. Developmental exposure to high concentrations of perchlorate (1000 µg/g) in Zebra finch resulted in greater begging intensity, decreased motivation for spontaneous movement (e.g., attempts to fly), and reduced capacity to wean themselves from parental care (Rainwater et al. 2008). flight attempts were also significantly reduced in birds exposed to lower concentrations (10 and 100 µg/g), and overall the proportion of perchlorate-dosed birds attempting flight was less than half that in the control group (Rainwater et al. 2008).

Juvenile European starlings exposed to Aroclor 1254 during development were also slower and more error-prone than controls in experiments testing: habituation, learning, cue selection, and memory (Zahara et al. 2015). Aroclor 1254 exposure resulted in significant increases in mass, fat, and moulting and decreasing plasma thyroid hormones over time. High dosed birds (1.05 µg Aroclor 1254/g) showed a delayed migratory orientation (Flahr et al. 2015).

6.2.3 Observations from wildlife

Many of the more serious adverse effects found in experimental studies (delayed/disrupted hatching, delayed metamorphosis, disrupted swim-bladder inflation, locomotion etc.) would result in predation or starvation in the wild. However, many impacts of thyroid disruption in environmental exposures are likely to be subtler. Animals in the wild (as humans) will be exposed to a multitude of lower levels of compounds with diverse targets, rather than single high doses as found in experimental studies. Without robust and sensitive methods or biomarkers to determine explicit thyroid effects it is not surprising it is so difficult to specifically ascribe thyroid disruption in wild animals.

Most wildlife studies that investigate possible thyroid disrupting effects focus on persistent organic pollutants. These generally measure contaminate load in the tissues and correlate them with circulating TH levels or more recently mRNA of thyroid related genes in tissues. However, these approaches are often confounded by intrinsic variability due to the animals varying age, sex, breeding stage, time of year, nutritional state, etc.

In 2011 Carr and Patino reviewed the literature on disruption of the hypothalamus-pituitary-thyroid (HPT) axis in wild amphibians and teleost by environmental contaminants, and found no evidence to clearly link contaminant-induced HPT alterations to impairments in wild population health (Carr & Patino 2011).

A recent review by Yu *et al.* (Yu et al. 2015) has summarize the effects of PBDEs on the thyroid and reproductive systems and the cross-talk between the two systems in fish, including lab and field data. Song *et al.* (reviewed in (Yu et al. 2015)) found decreased T4 and increased TSH in juvenile Crucian Carp (*Carassius auratus*) caught from a river close to an electronic waste (e-waste) site in Zhejiang, China compared to a control site, the 'e-waste' site fish had PBDE 400 times those in the control site. However, no population level effects were determined.

In wild urban gulls from Montreal, Canada, Técher *et al.* (Techer et al. 2016) investigated correlations between contamination with organochlorines (OCs) and polybrominated diphenyl ether (PBDE) flame retardants and thyroid related gene expression (mRNA) or circulating TH. The authors found positive correlations between liver concentrations of several polychlorinated biphenyls (PCBs), PBDEs as well as chlordanes and total plasma T4 levels. Hepatic concentrations of several PBDEs were negatively correlated with mRNA levels of ID3, TPO, and TRβ in the thyroid gland. Liver PCB (deca-CB) correlated positively with mRNA levels of NIS and TRα. In the brain, concentrations of most PBDEs were positively correlated with mRNA levels of organic anion transporter protein 1C1 and TTR, while PCBs positively correlated with expression of TRα and TRβ as well as ID2. The authors concluded that OCs and PBDE could be acting though several mechanisms (direct or compensatory), thus potentially perturbing the HPT axis of highly organohalogen-contaminated gull population (Techer et al. 2016).

Ideally, better integration between wildlife and laboratory studies, and improved models (*in silico*, *in vitro* and *in vivo*) could help understand (and prevent) possible adverse effects in wild animals.

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6.3 BACKGROUND PAPER 3: INTERPRETATION OF DATA FROM EPIDEMIOLOGY, EXPERIMENTAL STUDIES AND WILDLIFE DATA FOR THE IDENTIFICATION OF THYROID DISRUPTORS

6.3.1 Introduction

In this paper we describe issues that have an impact on the interpretation of data from epidemiological and experimental studies of thyroid disruption.

6.3.2 Data from human epidemiological and clinical studies

This section is intended to give some orientation as to the relevance of measures of thyroid function in epidemiological and clinical studies. It also summarises issues discussed in the literature that need to be taken into account for the interpretation of findings, especially relating to differences of the human thyroid system across life stages.

6.3.2.1 Thyroid hormone measurements and diagnostic criteria

Overt hyperthyroidism is defined in terms of suppressed TSH serum levels and free TH levels above the reference range. **Subclinical hyperthyroidism** is diagnosed when serum TSH is below 0.3-0.4 mU/L with free T3 and T4 levels within the population reference range.

Overt hypothyroidism occurs with TSH serum levels higher than 10 mU/L and T4 levels below the reference range. **Subclinical hypothyroidism** is diagnosed as "mild" with normal TSH serum levels between 4 and 10 mU/L and free T4 below the 10^{th} percentile of the reference range. The criteria for "severe" subclinical hypothyroidism vary somewhat. It is sometimes defined as TSH levels exceeding 10 mU/L. Other authors classify "severe" hypothyroidism with TSH in the normal range and free T4 levels below the 5^{th} percentile of the reference range (see the epidemiological studies on cognitive development in children discussed below).

There is no consensus on the upper normal range of TSH which leads to a controversy about the definition and clinical relevance of subclinical hypothyroidism. For references see **Background Paper 1 <BG1 adverse effects human>**.The prevailing method to diagnose thyroid illnesses uses limit values based on the distribution of THs or TSH in euthyroid disease-free individuals. The underlying assumption is that the limits derived from this curve are applicable to all people. However, TSH distribution and reference limits increase or decrease with age depending on iodine intake, and are unique for different ethnic groups (Surks and Boucai 2010). It has also been found that fT3 levels vary remarkably among different age groups and gender, decreasing with age, while fT4 remains relatively stable. The magnitude of the fT3 decrease may also be gender-specific (Kumari et al. 2015).

6.3.2.2 Variation of thyroid hormone levels across life stages

During the first trimester of pregnancy, maternal T4 is the only source of supply of TH to the fetus, until it can produce its own at approximately 16-20 weeks of gestation. TH and TSH levels during early pregnancy are exceedingly variable because of the enhanced requirements for thyroid hormone particularly during early pregnancy and because human chorionic gonadotrophin stimulates the thyroid in this period leading to a physiological decrease in TSH levels. The mother remains an important source of TH supply through to late gestation, contributing approximately 30% of the fetus' demand (reviewed by Ginsberg et al. 2007).

There are very low levels of TH in mother's milk. For this reason, newborns cannot anymore rely on mother's TH and have to synthesise their own supply. However,

newborns have quite low functional reserves of TH, and the reserves available in the adult are not yet established. This makes newborns more vulnerable to perturbations of the thyroid system. Several factors play a role (Ginsberg et al. 2007):

- The serum half-life of T4 is lower in neonates than in adults (3 days vs 7-10 days).
- The rate of TH replacement in neonates must therefore be higher than in adults.

The storage capacity of the neonatal thyroid is less than 1 day's worth of TH demand, compared to the adult gland which can store the demand of several months.

These factors need to be taken into account when considering acceptable levels of exposure to thyroid disrupting chemicals, but are sometimes ignored.

6.3.2.3 Endpoints used in human epidemiological studies and their interpretation

The endpoints most commonly measured in epidemiological studies include TSH, total T4 (TT4), total T3 and sometimes the free THs (fT3 and fT4).

Ethnicity, iodine intake, gender, age, and body mass index will all influence serum TSH, while pregnancy is associated with major changes in both TH and TSH concentrations (Koulouri et al. 2013). Hence each lab has to establish norms for their thyroid measurements and cohort/ exposure studies require large numbers to obtain sufficient statistical analytical power.

Changes in circulating binding proteins (e.g. elevated TBG in pregnancy) can also seriously confound interpretation of TT4 and TT3 concentrations. Another challenge concerns the measurement of free TH levels and methodological differences and limitations of commercially available immunoassays. The same sample may yield markedly discordant fT4 concentrations when run on different assay platforms (Koulouri et al 2013). Immunoassays have been reported to be influenced by the concentration of T4-bound transport proteins. The concentration of protein-bound T4 has been found to positively bias the fT4 readings generated by most immunoassays and measurement of free T3 would be expected to be similarly biased (Chevrier 2013).

6.3.2.4 Significance of altered TH levels in subclinical hyper- and hypothyroidism

By definition, subclinical hyper- or hypothyroidism describe altered TH levels without overt signs of disease. As such, for the individual patient, the interpretation of such alterations in terms of disease risk or prognosis is complicated.

However, **at the population level**, other interpretations are possible and necessary. In pregnant women, reductions in T4, accompanied by normal TSH levels (subclinical hypothyroidism) are associated with compromised cognitive development in their children (IQ loss) and other effects (see **Background Paper 1 <BG1 adverse effects human>**). These observations have to be taken into account when interpreting epidemiological studies showing associations between pollutant exposures and reduced T4 levels (as e.g. with mercury, certain arsenic compounds, certain organochlorine pesticides, perchlorate, thiocyanates, polyaromatic hydrocarbons and perfluorinated compounds.

Similarly, increases in TSH levels within the normal range that may arise in the wake of reduced TH levels are associated with increased risk of thyroid cancer (see **case study on thyroid cancer <CS TSH thyroid cancer>**).

6.3.2.5 Other factors with impact on thyroid hormone status: nutritional status, other illnesses and alcohol consumption

Some of the modifiable factors that may also have an impact on thyroid hormone status include nutritional status, non-thyroidal illnesses (NTIs) and alcohol consumption.

The reduction in leptin levels that accompanies malnutrition are thought to directly impair hypothalamic TRH secretion and induce central hypothyroidism (decrease in TH without concomitant increase in TSH. A role for excess endogenous glucocorticoids has also been postulated (Koulouri et al 2013).

Iodine is the major constituent of THs. It is remarkable that an element that is relatively scarce in the environment should play such an important role in embryogenesis, growth, metabolism, cognition, and adaptation to disease states. The sea is the main source of iodine; iodine-containing clouds are formed over the oceans, and deposited over the land by wet deposition. The iodine content of plants, crops, and animals in any specific geographical region depends on the retention of iodine in the soil. Iodine deficiency can pose a serious threat to the thyroidal capacity to synthesize thyroid hormones and about 1.5 billion people live in geographic areas of iodine insufficiency (Bianco et al 2014).

The thyroid is one of the organs with the highest selenium content because it expresses several specific selenoproteins including deiodinases that are implicated in thyroid hormone metabolism and others play an antioxidant defence role. The main source of selenium is proteinaceous foods (meat, fish, shellfish, offal, eggs, cereals, etc.), but bioavailability of the selenium they contain is variable and the selenium content of cereals is dependent on the selenium content of the soil where they are grown. The soils of most European countries have a low selenium content. Although only very severe selenium deficiencies appear to affect thyroid function, and namely T3 synthesis, selenium status appears to have an impact on the development of thyroid pathologies (Drutel et al 2013).

Changes in TH (especially T3) and TSH may be seen as early as 24 h after the onset of NTIs characterized by a variety of abnormal thyroid function patterns, which may change with progression or resolution of the underlying primary disorder. Reductions in TT4, and in particular TT3, are common even in mild NTI. Additionally, a number of drugs are recognised to increase serum TBG concentrations including oestrogen, raloxifene, tamoxifen, mitotane, fluorouracil, methadone and heroin. In contrast, androgens, chronic glucocorticoid therapy and nicotinic acid have all been shown to inhibit TBG synthesis (reviewed in Koulouri et al 2013).

6.3.3 Data from experimental studies with mammalian species

There are notable differences in the systemic regulation of thyroid hormones levels between commonly used rodent experimental models, particularly the rat, and humans. As illustrated in the **case studies** on perchlorate, fipronil and thyroid cancers (see section 5) quantitative species-specific differences in hormone synthesis and serum binding have been used to draw conclusions regarding the susceptibility of experimental models to TH perturbation and therefore the human relevance of experimental results.

Additionally, peripheral TH levels do not reflect tissue levels and therefore do not predict tissue responses, as illustrated by the **PBDE/perfluorinated compounds/PCB case study** (section 5.4).

6.3.3.1 Species differences in relation to the thyroid system and their relevance in interpreting the outcome of animal studies

The HPT axis and basic processes of TH synthesis and release are qualitatively similar across species. The negative feedback loop between circulatory TH levels and synthesis of thyrotropin-releasing hormone (TRH) by the hypothalamus, and thyrotropin (TSH) by the anterior pituitary is a primary signalling pathway to maintain homeostasis. There are however notable quantitative species-specific differences in hormone synthesis and serum binding.

When released to the bloodstream, the two thyroid hormones T3 and T4 are bound to transport proteins that protect them from metabolic degradation and reduces their

elimination via the kidneys. In humans, approximately 68% of the total circulating T4 (TT4) is bound to thyroxine-binding globulin (TBG), a specific, high affinity transport protein. The remainder is bound to less specific transport proteins such as albumin and transthyretin (TTR), with less than 1 percent existing as the free (biologically active) hormone (fT4) (Lewandowski et al. 2004; Fisher et al. 2012). TBG is found in primates, as well as in dogs and certain ungulates. In rats however, it is found in the serum of the young peaking at about one month of age, then drops to very low levels by two months, before returning to higher levels in old age, beginning at about seven months and reaching levels that are approximately 25% of the peak post-natal levels by about 20 months of age. Between the ages of two to seven months typically used in subchronic toxicology studies, levels of TBG are therefore hardly detectable. Instead, T3 and T4 are bound to primarily TTR and to some extent albumin that both have lower affinity. In humans, TBG-bound TH acts as a stable reserve that may be used if additional amounts of thyroid hormones are required. In species in which TBG is not the major T4 carrier, the free fraction of the T4 is larger than in humans and total T4 half-life is shorter (around 24h in rats, versus 5-6 days in humans) (Lewandowski et al 2004). This increased clearance is compensated by higher production of THs with corresponding higher basal TSH levels.

This is also reflected in species-specific differences in thyroid histology. Thyroid hormone production requires the transport of iodide into thyroid follicular cells by the sodium iodide symporter (NIS) where it is oxidised to iodine by the enzyme thyroglobulin peroxidase (TPO). Iodine is then coupled to tyrosine residues on the thyroglobin molecule (TG) and stored within the lumen, a cavity inside the thyroid follicle, in the form of a viscous substance called colloid. This reserve pool of TH precursor can be called upon in response to decrease serum TH levels. In the rat, thyroid follicles tend to be smaller and contain less colloid than primate follicles.

These differences, as well as the examination of T3 and T4 and, particularly, TSH responses to perchlorate in rats in comparison with humans (see case study) has led to the suggestion that humans have a greater potential for adaptation after exposure to chemicals, without suffering changes in levels of serum thyroid hormones. For these reasons, the toxicological effects on thyroid function that derive from increased thyroid hormone catabolism in the rat have often been considered irrelevant to humans.

However, newborns have lower storage capacity (see above).

However, even though TBG is the major and specific transport protein of thyroid hormones (TH) in humans, it has recently been shown not to be the physiologically most relevant (Alshehri et al. 2015). The most physiologically relevant carrier protein in humans is also TTR (**see fipronil case study**, section 5.1). Furthermore, the differences in half- lives of the active hormone T3 between rats and humans are much smaller than for T4, as T3 half-lives for humans are 22-24 h (Jonklaas et al 2015) and around 6h in rats (Lewandowski et al 2004). It is also important to point out that the newborn human has lower stored thyroid hormone and the half-life of its T4 is much shorter than in adults.

6.3.3.2 Commonly used endpoints for measuring thyroid disruption and their interpretation in terms of adversity and thyroid disruption

Repeated-dose toxicity as well as reproductive and developmental toxicity studies include endpoints relevant to identifying thyroid hormone mediated effects. The studies involving exposure of developing animals are particularly important in this context: TG421/422 reproductive/developmental toxicity screening study), TG 443 (extended one-generation study) and TG426 (developmental neurotoxicity) (see **Background Paper 4** below on the gaps in current testing methods).

Common difficulties that arise when interpreting observed changes on these endpoints are summarised in the next section and illustrated in all case studies.

6.3.3.3 Adversity of TH reductions and enigmatic effect profiles and difficulties of their interpretation

The **case study** on fipronil (see section 5) describes the debates that surround interpretations of reductions in T3 and T4 levels, and whether these should be judged as "adverse" per se.

The **case studies** on Mancozeb and PBDEs/perfluorinated compounds/PCB (section 5) illustrate some effect profiles that remain unexplained and highlight the difficulties in interpreting experimental studies reporting effects on circulating TH levels.

Mancozeb is thought to exert its thyroid disrupting effects through the same mode of action as PTU (inhibition of PTO). However, whereas the marked maternal reductions in T4 following PTU exposure lead to severe developmental neurotoxicity in offspring, hypothyroxinemia following maternal exposure to mancozeb does not lead to significant effects in offspring behaviour or acoustic startle response. Several other DNT studies investigating rodent offspring from hypothyroid dams have also shown that adverse behavioural outcomes are not always present. This is for instance the case with fipronil and perchlorate (see case studies).

The majority of reported developmental PTU studies use very higher doses of PTU than those used in mancozeb studies. It remains to be seen whether higher doses of mancozeb would induce neurobehavioural effects. PTU has other modes of action than just TPO inhibition, including inhibition of deiodinase activity. Decreases in circulating T4 levels (due to TPO inhibition by mancozeb) could have led to upregulation of peripheral deiodinase activity in the offspring's brains and compensated for the low circulating T4 levels by providing enough T3 for normal brain development. Since much brain development occurs postnatally in rats and the offspring in mancozeb studies were not hypothyroid in that postnatal period (due to limited milk transfer), the prenatal hypothyroxinemia may not be severe enough to disrupt brain development. Finally, it is also possible that neurobehavioural assessment methods are not suited for the detection of subtle effects in the brain.

In rodent studies, polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and poly- and perfluorinated alkyl substances (PFASs) reduce total and free serum T4, but without corresponding increases in TSH, a pattern described as "enigmatic". In order to shed light on the significance of altered circulatory TH levels, the accompanying case study compared the effects of PBDES, PCBs and PFASs and their metabolites on the Hypothalamus-Pituitary-Thyroid axis, binding to proteins involved in peripheral TH transport, the regulation of intracellular levels of THs, binding affinity for thyroid receptors (TR), and downstream TH-mediated effects in some of the best studied organs (liver, brain, testes). The evidence available reveals both that these three classes of compounds can act, sometimes in opposite direction, centrally on the HPT axis, on tissue levels of TH by binding to peripheral transport proteins, cellular transporters or disrupting deiodinase action, and ultimately on TR-mediated genes. There is also clear evidence that one substance can elicit different responses in different tissues. It is therefore clear that interpretation of changes in peripheral TH levels is not 'one size fits all'. Similar patterns of effects peripherally may mask the complexity of concurrent disruption of TH action on several regulatory processes centrally, peripherally and in different tissues.

6.3.4 Data from experimental studies with non-mammalian species

The thyroid system was long considered the preserve of vertebrates. However, more recently it has been shown that TH functions also occur in invertebrate chordates, echinoderms, and possibly molluscs. Thyroid endocrinology is generally well conserved across vertebrate taxa, with similarities in thyroid synthesis, metabolism and mechanism of action. Therefore, chemicals which disrupt the thyroid system by targeting thyroid receptors as agonists or antagonist, thyroid hormone transport proteins, thyrotropin releasing hormone (TRH), or alter thyroid synthesis or metabolism could impact a wide range of vertebrate species. The following section summarises key points from

Background Paper 2, "The thyroid system in fish, amphibians and invertebrates – a comparison with mammalian species" (see previous section)

6.3.4.1 Commonalities and differences of thyroid systems

Iodine is the major constituent of THs. Iodide is actively transported into thyroid follicular cells via the NIS, where it is conjugated to TG, a process catalysed by TPO. TG had until recently only been investigated in mammals. However, TG orthologs have now been identified in fish and amphibians, and NIS and TPO homologs in amphibians, fish and birds**.** As in mammalian species, TH release from thyroid follicles is regulated by pituitary TSH and the activity of the HPT axis is regulated by negative feedback by circulating THs. In mammals, TRH controls the release of TSH. However, although present in non-mammalian vertebrates, it does not always act as a TSH-releasing factor, whereas corticotropin-releasing hormone (CRH) appears to be a potent stimulator of hypophyseal TSH secretion. A recent review suggests additional close interactions between adrenal/interrenal and thyroidal axes, highlighting that corticoids also affect the expression of deiodinases and thyroid hormone receptors. In humans, 3 iodothyronine deiodinase (ID) enzymes are involved in converting, recycling and degrading T4 and T3. Homologs of ID genes have been found in non-mammalian vertebrates such as xenopus, zebrafish, chicken and alligator.

In humans, TBG had been considered 'more important' than the other transport proteins due to its higher affinity. However, due to the different dissociation rates TTR is actually responsible for the majority of TH delivery to tissues. Alshehri et al. neatly use the metaphor of 'Goldilocks and the three bears' to describe the relationship, in that, TBG binds THs too tightly to deliver significant quantities under healthy conditions; albumin binds THs too loosely; and TTR has 'just right' dissociation rates (Alshehri et al. 2015). Similar dissociation rates for TTR have been found across species (Richardson 2007; Chang et al. 1999). TBG is not found in all species, with albumin being found most frequently as the binding protein in a survey of 150 species of adult vertebrates. In many vertebrate species, the production of certain thyroid binding proteins is transient and related to specific developmental stages. While adult birds and many adult mammals have TTR as their main form of thyroid binding transport protein, in fish and amphibians, hepatic TTR synthesis is primarily found during early development, metamorphosis and smoltification (salmonids). Only one of the transport proteins, TTR, is synthesised in the brain of mammals and involved in transporting TH across the blood-brain barrier. TTR is also synthesised in the choroid plexus of non-mammalian terrestrial vertebrates (birds, reptiles) but not aquatic vertebrates (fish, amphibians). TTR was first described in mammals as T4 binding protein. However, it seems mammals are the exception among vertebrates in respect to the function of TTR, as in teleost fish, amphibians, reptiles and birds TTR preferentially binds the active form T3. It is also important to note that total T4 and total T3 levels in blood vary between classes of vertebrates.

Although much of the machinery of the thyroid system is fairly-well conserved across taxa, there are some striking physiological differences. For example, teleost species can have heterotopic thyroid tissue, often with dispersed follicles, whereas, reptiles, birds and amphibians generally have medial or paired glandular structures.

6.3.4.2 Use of amphibians and fish for the identification of thyroid disruptors

TH has a well-established key role during early development in vertebrates. One of the most notable models for studying disruption to this function is amphibian metamorphosis. The most sensitive and robust endpoint in metamorphic xenopus assays (OECD screening test, TG 231) is generally considered to be disruption to thyroid histopathology (Pickford 2010). More recently several studies have integrated additional endpoints (e.g. molecular, TH measures) alongside traditional histopathological and developmental scores to help eluate the mechanism/target of disruption. These studies have shown that modulations of gene expressions of components of the iodine machinery

are often more sensitive effect markers. The same is true for genes related to TH metabolism and for gene expression in the brain.

There are currently no specific OECD tests for thyroid disruption in **fish or birds**. Recent studies suggest several possible endpoints for thyroid disruption in fish and birds (see background paper 2 in this ection).

6.3.4.3 From the laboratory to the field

To anticipate the effects of thyroid disrupters in the field, the ecotoxicological effects that are considered relevant are those likely to affect populations. As such, the main focus is on effects affecting the growth, survival or reproduction of species. As illustrated in background paper 2, guideline studies have been validated for the screening of thyroid disrupting properties. The endpoints included were therefore selected on the basis of their sensitivity rather than their relevance to field conditions. While some of the more serious adverse effects found in experimental studies such as delayed/disrupted hatching or delayed metamorphosis may be included, other subtler effects are likely to be disregarded in the absence of documented effects on growth, reproduction or survival. It is unclear whether the effects on fish or bird behaviour described in background paper 2 would be considered despite their relevance potential relevance to predation. In turn, the impact of increased predation on populations will depend on the life history traits of the species considered as well as its ecological environment.

Moreover, there are questions related to the ecological relevance of experimental animal. It is clear that endpoints related to thyroid disruption have only been validated in a few taxonomic groups. Furthermore, within these taxonomic groups, test species are generally selected on the basis of experimental and economic considerations rather than their ecological relevance to a given environmental context. These considerations are not however specific to thyroid disrupters.

6.3.4.4 Endpoints for populations and ecosystems

Most wildlife studies that investigate possible thyroid disrupting effects focus on persistent organic pollutants. These generally measure contaminate load in the tissues and correlate them with circulating TH levels or more recently mRNA of thyroid related genes in tissues. However, these approaches are often confounded by intrinsic variability due to the animals varying age, sex, breeding stage, time of year, nutritional state, etc. (see background paper 2 in this section).

Many of the more serious adverse effects found in experimental studies (delayed/disrupted hatching, delayed metamorphosis, disrupted swim-bladder inflation, locomotion etc.) would result in predation or starvation in the wild. However, many impacts of thyroid disruption in environmental exposures are likely to be subtler. Animals in the wild (as humans) will be exposed to a multitude of lower levels of compounds with diverse targets, rather than single high doses as found in experimental studies, as well as other environmental pressures on their habitat, physical climate change or disease. Without robust and sensitive methods or biomarkers to determine explicit thyroid effects it is not surprising it is so difficult to specifically ascribe thyroid disruption in wild animals.

Nonetheless, increased predation itself, even without marked effect on population size, may have subtler effects relevant to the conservation of biodiversity. Chemical exposures may result in the selection of a resistant phenotype, thereby potentially reducing the genetic diversity of an exposed population in the wild. Inbreeding can be an important determinant of the vulnerability of populations to environmental stressors. Evidence for an interaction between inbreeding and chemical exposure in the wild is extremely limited. Studies on the Florida panther have found that this species is now significantly inbred and animals also contain high levels of a range of EDCs, including mercury and PCBs. However this has been linked to the known reproductive effects of these chemicals at the detected levels (Brown et al. 2009).

6.3.5 Controversies

The main controversies in interpretation of experimental data are related to predicting effects on human health, namely; the human relevance of the rat model, whether effects on TH alone without any effect on TSH are adverse, and finally whether TH decreases due to hepatic clearance can be considered indirect effects.

The rat model has been labelled as problematic with regards to its human relevance (see perchlorate and fipronil case studies. The rat, due to species differences in T4 half- lives and in carrier proteins, has been viewed as particularly sensitive to thyroid disturbances. However, assumptions about the importance of such quantitative difference were based on interspecies differences in the half-lives of T4. The half-lives of the active hormone T3 are much more similar in both species. Further, TBG was thought to be the main physiologically relevant transport protein in humans whereas it is hardly detected in adult rats. However, it was recently shown that TTR is the main physiologically relevant distributor in humans also. It is therefore timely to reconsider the significance of these species differences when interpreting experimental data.

Further, the interpretation of effects on TH in the absence of corresponding effects on TSH has been questioned in terms of their adversity. Such effects have been seen with PBDEs, PCBs and PFASs (see case study). The fact that peripheral TH levels are poor predictors of TH levels and actions in tissues is now well recognized. Another interesting perspective on whether T4 decreases should be considered adverse comes from clinicians. Subclinical hypothyroidism is defined as T4 $<10^{th}$ percentile (severe is defined as $<5^{th}$ percentile) but without increased TSH. It is therefore difficult to generalise conclusions on the basis of these endpoints alone. This also illustrates that the relevance of animal models should be interpreted in relation to the relevance of specific pathways.

Increased TH clearance due to liver effects is often considered secondary or an indirect effect, and therefore not a primary endocrine disrupting effect (see fipronil case study). The assumption here is that it would possible to protect against TH insufficiency by regulating in relation to a "liver threshold" below which hepatic enzymes are not induced and consequently TH insufficiency cannot occur. On the other hand, an endocrinological perspective would not class TH clearance as a "secondary" effect, and rather evaluate this in the context of a balance between hormone-synthesising and –removing processes. Additionally, activation of liver enzymes by a chemical could be reversible; however, effects on target tissues (e.g. fetal brain) of thyroid hormone insufficiency during development caused by activation of liver enzymes in a pregnant animal would not be reversible.

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6.4 BACKGROUND PAPER 4: CURRENT OECD TEST METHODS FOR THE IDENTIFICATION OF THYROID DISRUPTORS, THEIR GAPS AND POSSIBILITIES OF IMPROVEMENTS

6.4.1 Introduction

In this paper we map relevant assays within the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals (here referred to as the OECD Framework 2012) and identify the gaps in these validated test methods in relation to the identification of thyroid disrupting effects. This overview is structured according to the different Levels detailed in the OECD Framework (Levels 1-5), dealing with mammalian assays and non-mammalian assays separately.

We also summarize currently debated suggestions for improvements in order to better identify substances causing adverse effects via disruption of the hypothalamic-pituitary-thyroid-axis.

6.4.2 Existing data, non-test methods, in vitro assays with mechanistic data (OECD framework Levels 1 - 2)

Level 1 information of the OECD Framework deals with "Existing Data and non-test information". There are ongoing activities aimed at improving non-test information on thyroid disrupting effects. A Quantitative Structure Activity Relationship (QSAR) computer model for predicting TPO inhibition is presently being developed in collaboration between the US EPA and the Technical University of Denmark (Rosenberg et al 2016). QSAR models for predicting binding to CAR/PXR receptors (possibly leading to increased liver catabolism of thyroid hormones) are also being developed (Rosenberg et al 2017). Predictions from these types of QSAR screenings are already in use in a tiered approach to prioritize potential thyroid disrupting chemicals for further evaluation.

Level 2 of the OECD Framework details *in vitro* assays for selected endocrine modalities. There are currently no OECD *in vitro* assay test guidelines assessing modalities relevant to thyroid disruption.

6.4.3 In vivo tests (OECD framework levels 3-5)

Test methods in mammalian species **Level 3** include in vivo assays that provide data about specific endocrine mechanisms or pathways. Level 3 mammalian toxicology assays for the screening of endocrine disrupting effects include the two screening assays for estrogenic and androgenic/anti-androgenic chemicals, namely the Uterotrophic and the Hershberger assays (OECD TG 440 & 441). No thyroid related endpoints are mandatory in these test guidelines, but T3 and T4 measurements are optional in the Hershberger assay.

Levels 4 and 5 mammalian assays include repeated dose toxicity studies, as well as those investigating reproductive and developmental toxicity. **Level 4** *in vivo* assays provide some data on adverse effects on endocrine relevant endpoints, whereas levels 5 are *in vivo* assays provide more comprehensive data on adverse effects on endocrine relevant endpoints, and over more extensive parts of the life cycle of the organism. A complete list of studies in the OECD Framework can be seen in **annex 1**. Thyroid related endpoints in Level 4 and 5 TGs (mammalian) are presented in Table 1, below. The TGs have here been divided into two groups, one where exposure occurs only in adult animals and one where both adult individuals as well as animals exposed during foetal and postnatal development are examined. Because of the importance of thyroid hormones during brain development, especially the studies involving exposure of developing animals are important in this context.

OECD Test Guideline	Study name	Thyroid endpoints measured in the guideline
ADULT EXPOSURE ONLY		
407	Repeated Dose 28-Day Oral Toxicity Study	After 28 days of exposure, assessment of thyroid gland histopathology is mandatory, thyroid gland weight, T3, T4 and TSH levels are optional
408*	Repeated Dose 90-Day Oral Toxicity Study in rodents	After 90 days of exposure, assessment of thyroid gland histopathology is mandatoy
451-3	Chronic toxicity and carcinogenicity studies	After 2 years of exposure; assessment of thyroid gland weight, and histopathology (including thyroid tumours) is mandatory
STUDIES WITH DEVELOPMENTAL EXPOSURE		
414	Prenatal developmental toxicity study	Dams and offspring are sacrificed on the day before birth. At present no thyroid related endpoints are included in guideline, but work is ongoing in the OECD to include mandatory TH measurements in this guideline.
415	1-generation reproduction toxicity study	No assessment of thyroid endpoints is included in this guideline, but this test guideline has essentially been replaced by TG 443
416	2-Generation reproduction toxicity study	Thyroid gland histopathology in parental animals (P and F1) is mandatory.
421	Reproductive screening test	This guideline has very recently been updated to include TH measurements. Mandatory; T4 measurements in blood from PND 13 offspring and adult males (P) at study termination. If relevant T4 is also measured in pups on PND 4 and dams on PND 13. Assessment of other hormones (T3, TSH) is optional, as well as thyroid gland weight and histopathology.
422	Combined 28-day/reproductive screening assay	As in TG 421
426	Developmental neurotoxicity study	No assessment of thyroid endpoints *per se*, but investigation of adverse effects on the developing brain.
443	Extended One-Gen Repro-Tox Study	Some measurements of thyroid hormone levels are mandatory; T4 and TSH levels in F1 offspring from cohort 1A at termination (PND 22) Furthermore, T4 measurement in surplus offspring on PND 4 are optional. At necropsy, thyroid gland weight and histopathology are assessed and in cases where the DNT cohort is included, some limited investigations of adverse effects on the developing brain are also conducted.

* TG 409 (repeated dose 90 day oral tox in non-rodents) includes thyroid weight and histopathology, TG 411 & 413 (subchronic dermal & inhalation) includes thyroid histopathology

Table 1 *OECD test guidelines in mammals, included in the conceptual framework (OECD 2012) with focus on assessing thyroid disruption. In the next revision of OECD Guidance Document 150 (probably finalised ~2018), TG 409, 411, and 413 will most likely be included whereas TG 415 will not*.

Furthermore, the US EPA has TG for the male and female pubertal rat assay that includes thyroid gland weight, thyroid histopathology as well as TSH and T4 measurements.

6.4.4 Summary of key 'gaps' in mammalian test species

In the presently available REACH registrations, often very little information is available on a chemical's possible effects on the thyroid hormone system. Until recently, none of the guidelines included mandatory assessment of thyroid hormone levels, so in many cases the only available information regarding thyroid toxicity was thyroid gland histopathology from repeated dose toxicity studies. As seen in many of the presented **case studies**, and as also reviewed by Pickford (2010), several chemicals can significantly lower circulating T4 levels at exposures where no adverse histopathological effects on the thyroid gland are seen. Some compounds are even seen to have "enigmatic" patterns where T4 levels are decreased but no adverse effects on the thyroid gland (or TSH levels) are seen even at quite high doses. In the review by Pickford (2010) thyroid disrupting chemicals were grouped by their thyroid disrupting mode of action, and the author investigated which endpoints seemed to be the most sensitive in both amphibian and rodent models. It was shown that for chemicals interfering with thyroid hormone transport, metabolism and elimination, (exemplified by PCBs, the PBDEs and phenobarbital) significant reductions in thyroid hormone levels are often seen at exposure levels where no adverse histopathological effects or TSH increases are seen. This means that negative findings on thyroid histopathology do not necessarily eliminate a concern for thyroid disruption.

This "gap" is presently being dealt with, by including assessment of thyroid hormone levels into more test guidelines (TG 407/408, TG 421/22, TG 443, and ongoing work regarding TG 414). All of these TGs are included in the REACH Information Requirements, and therefore much more information on which chemicals affect thyroid hormone levels in rats will be available during the next decade.

One issue that is presently very poorly dealt with in the guideline studies is how to assess the adverse downstream effects of such alterations to the thyroid hormone levels. Thyroid hormones control many important functions in the adult organism, but the main concern in human health risk assessment regarding thyroid disrupting chemicals is that chemical exposure may interfere with the proper delivery of thyroid hormone to the developing brain, which could consequently cause neurotoxicity.

At present, no guideline studies are designed to fully assess this issue. The TG 426 DNT study and the TG 443, EOGRTS (with DNT cohort) are at present the studies best designed to address this, but both have shortcomings. In the TG 426, no thyroid hormone measurements are performed, and in TG 443 often the DNT cohort is not included, and even when it is, fewer neurotoxicity endpoints are assessed than in the TG 426. Furthermore, several behavioural studies performed according to these guidelines have not been able to show any adverse effects in behaviour or on brain histopathology, even in severely hypothyroxinemic animals. This has lead scientists in the field of neurotoxicology and thyroid disruption to suggest that new endpoints which are more sensitive to perinatal thyroid disruption should be added to these guidelines, in order to better assess adverse effects on neurodevelopment (OECD 2006, Harry et al 2014).

From **Background Paper 1** (see this section) it is obvious that thyroid disruption has negative impacts on the cardiovascular system, on lipid metabolism and peripheral neuropathies in the human. Endpoints relevant to these effects are currently missing altogether.

6.4.5 Non-mammalian test species

The only OECD test guidelines using non-mammalian animals which specifically detect thyroid endpoints are TG 241 and TG 231. These assays both employ *Xenopus laevis* frogs from tadpole to juvenile stage (Table 2). The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters (as revised in 2012) includes a large number of non-mammalian models at different levels. However, many of these assays are either still under development or do not include thyroid related endpoints.

Level 3 *In vivo* assays providing data about selected endocrine mechanism(s)/pathway(s). The Amphibian Metamorphosis Assay (TG 231) is currently the only TG with any thyroid specific endpoints at this level. The main thyroid related endpoints considered are comparative metamorphic stage (delayed/precocious compared to controls, hind limb length), growth (weight, snout-vent length) and thyroid histopathology. Unlike in the rodent model T4 and TSH are not measured in TG 231.

Level 4 The Larval Amphibian Growth and Development Assay (LAGDA) (TG 241) is currently the only non-mammalian TG with thyroid specific endpoints at this level. TG 241 covers an extended time period compared to TG 231, and includes sex ratio and sexual development (gonad histopathology) alongside the traditional growth, metamorphic and thyroid histopathology endpoints.

Level 5 There are no non-mammalian TGs that include specific thyroid endpoints at this level.

Test Guideline name and no.	**Species** **Model**	**Conceptual Framework Level**	**Life stage(s)** **Embryo (E)** **Juvenile (J)** **Adult (A)** **Generation: (F0, F1)**	**Specific thyroid endpoints: T4, TSH, Histopathology**	**Non-specific endpoints that could indicated thyroid disruption: altered behaviour (B), development (D), growth (G)**
241: The Larval Amphibian Growth and Development Assay (LAGDA)	*Xenopus laevis*	Level 4	J (F0)	Thyroid histopathology (larval sub-sample) Time to metamorphosis	B D G
240: Medaka Extended One Generation Reproduction Test (MEOGRT)	*Oryzias latipes* (fish)	Level 5	A E J (F0, F1)	-	B D G
236: Fish Embryo Acute Toxicity (FET) Test	*Danio rerio*	-	E (F0)	-	D
234: Fish Sexual Development Test	*Gasterosteus aculeatus* *Oryzias latipes* *Danio rerio*	Level 4	E J (F0)	-	B D G

231: Amphibian Metamorphosis Assay (AMA)	*Xenopus laevis* (frog)	Level 3	J (F0)	Thyroid histopathology (larval sub-sample) Time to metamorphosis	B D G
230: 21-day Fish Assay	*Pimephales promelas* *Oryzias latipes* *Danio rerio*	Level 3	A (F0)	-	B
229: Fish Short Term Reproduction Assay	*Pimephales promelas*	Level 3	A (F0)	-	B
215: Fish, Juvenile Growth Test	*Oncorhynchus mykiss*	-	J (F0)	-	B D G
210: Fish, Early-life Stage Toxicity Test	e.g. *Oncorhynchus mykiss* *Oryzias latipes* *Pimephales promelas* *Danio rerio*	-	E J (F0)	-	B D G
206: Avian Reproduction Test	*Anas platyrhynchos* *Colinus virginianus* *Coturnix japonica*	Level 4	A E J (F0, F1)	-	B D G

Table 2 *OECD test guidelines, using of non-mammalian test species which could be subject to thyroid disruption, from OECD Guidelines for the Testing of Chemicals, Section 2 'Effects on Biotic Systems' (http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-2-effects-on-biotic-systems_20745761)*

6.4.6 Summary of key 'gaps' in non- mammalian OECD test species

The preceding description allows us to summarise the gaps in non-mammalian tests as follows :

- There are no direct measures of TH – T3/T4 or TSH (little link with current mammalian endpoints)
- Less integration of multiple endocrine endpoints in non-mammalian tests
- There are no set neurological or behavioural end points
- Embryo culture techniques are not covered
- Maternal transfer is not covered in non-mammalian tests

- There is no avian model

6.4.7 Possibilities of addressing gaps in mammals and non-mammalian species

6.4.7.1 In vitro test methods

OECD has published a scoping document (OECD 2014) on *in vitro* and *ex vivo* assays for the identification of chemicals that can interact with the various elements of the thyroid system. The scoping document groups *in vitro* and other non-animal assays by utilising a scheme developed by Murk *et al.* (2013) which assigns test methods to organisational aspects and target sites of the thyroid system, as follows:

- Central regulation of thyroid hormone synthesis
- Thyroid hormone synthesis
- Hormone secretion and transport
- Hormone metabolism and excretion
- Local cellular concentrations
- Cellular responses
- Short-term assays that integrate multiple modes of action
- Integrative cellular assays

For each of these groups of assays ("blocks"), the document assesses their readiness to be taken forward to the validation process. On the basis of this assessment, the OECD Scoping Document identified five assays as top candidates for pre-validation in the short term:

- Thyroid peroxidase inhibition,
- Transthyretin binding,
- Thyroxine-binding globulin binding,
- Thyroid transmembrane transporter,
- Thyroid receptor transactivation.

Several other assays were judged to be ready for validation in the medium to long term: Thyrotropin-releasing hormone receptor activation, thyroid stimulating hormone receptor activation, sodium-iodide symporter (NIS) inhibition, deiodinase up- or down-regulation, zebrafish embryo and thyroid gland explant cultures.

Following on from the OECD scoping effort, the next logical step would be to determine whether these assays can be supported for optimisation and inter-laboratory validation, as part of a test method validation process.

However, a key question with this issue of *in vitro* and *ex vivo* assays is that there is very little information about the relationship between interference with these single molecular initiating events, circulating levels of thyroid hormone, and downstream "adverse" effects. Moreover, there is virtually nothing about the impact of having multiple MIEs targeted at once, and there are single chemicals that have this potential. For example, mancozeb can activate liver enzymes reducing serum T_4, but also can inhibit TPO. What is the consequence of this? How would this be altered in the setting of low dietary iodine or perchlorate exposure (or smoking)? Thus, even if these in vitro endpoints are established and validated, interpretation will continue to be difficult.

6.4.7.2 Addressing the gaps in mammalian test systems

As exemplified in the **mancozeb case study** (section 5) the presently used test methods for assessing developmental neurotoxicity (OECD TG 426 and the DNT cohort of TG 443) may be inadequate for assessing alterations in brain development caused by thyroid disruption.

Presented below (Table 3) is an overview of additional endpoints which could be considered relevant to include in the two guidelines investigating developmental neurotoxicity, and possibly also in the developmental screening study (TG 421/22).

These endpoints all seem to be more sensitive to the effects of thyroid disrupting chemicals, than the endpoints which are presently included in these guidelines (brain weight and histopathology, and assessment of animal behaviour).

Endpoint	Comments	Ref
Neuronal Migration (subcortical heterotopias)	Elements of cognitive deficits are linked to neuronal migration disorders. Lissencephaly, cortical band and subcortical heterotopias, disorganization of cortical layers all are associated with cognitive deficits and linked to thyroid hormone during human (and rodent) development. These endpoints have been investigated in PND 14 offspring in developmental toxicity studies and could therefore be included in both TG 421/22, TG 443 and TG 426.	Gilbert et al 2014, Korevaar et al 2016, Yu et al 2015, Tamijani et al 2015
Neuronal Proliferation	Thyroid hormone plays an important role in neuronal proliferation during early development and also in adults. However, this effect is not observed uniformly through the brain. Rather, thyroid hormone alters neuronal proliferation in specific cell populations at specific times during the life cycle.	Preau et al 2015, Kapoor et al 2015, Ernst et al 2015, Remaud et al 2014, Ernst et al 2014
Oligodendrocyte differentiation	Oligodendrocytes are myelin forming cells in the central nervous system. Hypomyelination is causative for neurological and cognitive impairment in humans. Multiple Sclerosis is a myelin-degenerating disorder in humans with severe repercussions. Thyroid hormone is important in oligodendrocyte differentiation and myelination during development and it is essential in remyelination after injury in adults. Thyroid hormone is linearly related to oligodendrocyte number in rodents and assessment of this endpoint could possibly be developed into be a useful endpoint in guideline studies.	Dugas et al 2012, Tamiji et al 2015, Noda et al 2015, Lopez-Espindola et al 2014, Sawano et al 2013, Silvestroff et al 2012, Picou et al 2012, Dell'Acqa et al 2012
Cerebellar histogenesis	Cerebellar development is well-known to be dependent upon thyroid hormone. This is linked to human neurological disorders and specific deficits in cognitive domains. This would be a relatively simple endpoint to capture in regulatory studies.	Picou et al 2012, Dezonne et al 2015, Ortiga-Carvalho et al 2014, Faustino et al 2014, Fauquier et al 2014, Ibhazehiebo et al 2011

Table 3. *Additional neurotoxicity endpoints, which could be considered relevant to include in guidelines investigating adverse effects on offspring perinatally exposed to thyroid disrupting compounds.*

Furthermore, including gene expression of thyroid regulated genes in key tissues (including different areas of the brain) in the studies dealing with developmental exposure (TG 421/222, TG 426 and TG 443) would help inform us on the mechanism

occurring after chemical exposure. Moreover, analysis of perturbation of some of these DNT-related endpoints (i.e., neural progenitor cell proliferation, migration and differentiation toward oligodendrocytes), resulting from thyroid hormone decrease, has been promisingly carried out on human *in vitro* models (Moors et al., 2009; Fritsche et al., 2005; Schreiber et al., 2010).

The gold standard of endpoints of thyroid "disruption" would be to identify and validate easily captured endpoints of thyroid disruption that would be sensitive and specific for a thyroid MOA and be reflective of adverse outcome. Having such a battery would allow one to discriminate between a benign reduction in serum T_4 or an adverse effect even in the absence of a change in serum T_4. These would have to be debated, identified and validated. A possible strategy to accomplish this is base on the CLARITY-BPA study that is a collaboration between the US NIH, FDA and NTP (Heindel et al. 2015; Schug et al. 2013). In brief, the core study would be a guideline, GLP-compliant study (the TG to be agreed upon by an international committee). The TG would be completed and tissues collected as prescribed. Guideline endpoints would be captured by the core lab. In addition, however, selected tissues would be coded and three samples of each tissue would be shipped to participating labs through the EU. Each putative thyroid endpoint - agreed upon previously by committee – would then be captured by three independent labs and sent (coded) to the core facility that would break the code and analyse the data. This would significantly streamline the identification and acquisition of endpoints. These endpoints would have to be tailored to the TG in relation to timing and duration of chemical exposure and the timing of sacrifice.

It would also be timely to develop endpoints relevant for assessing the effects of thyroid disruption on the cardiovascular system and on lipid metabolism in mammalian test organisms.

6.4.7.3 Addressing the gaps in non-mammalian test species

One area for broadening non-mammalian TG endpoints to link with mammalian TGs, would be the addition of TH measures in thyroid tissue or plasma. However, the timing and location of sampling would need to be considered. For example, disruption to thyroidal T3 and T4 has been shown to be more sensitive than plasma T4 in 7-day exposed metamorphic Xenopus (Tietge et al. 2013). It would also be important to consider the balance of information gained or lost (or number of additional animals required). For example, would it be possible to 'split' thyroid tissue between TH and histopathological analysis in Xenopus (TG 231, 241), or would the tissue be too small? Similarly, plasma samples are already taken in many of the current TG for measuring endocrine effects in fish (e.g. TG 229, 230, 234 and 240) and amphibians (i.e. TG 241) for vitellogenin (female specific yolk protein) analysis (as a biomarker oestrogenic action). In smaller species (medaka, zebrafish) or small/young animal(s) plasma volume might be too low to measure multiple biomarkers/endpoints. However, technical advances are enabling highly sensitive enzyme-linked immunosorbent assay (ELISA) to measure vitellogenin in fish mucus (from external swabs). Therefore, if TH measures are considered valuable there could be ways of including them into non-mammalian TGs.

Traditionally, in the Xenopus model, the metamorphic state was considered the most sensitive window for chemical disruption. More recently, the important role of thyroid system in early embryos of frogs and fish has been highlighted (Schnitzler et al. 2016; Thienpont et al. 2011; Duarte-Guterman et al. 2010).

Owing to their shorter exposure period, reduced ethical concerns and lower cost, embryo culture techniques are increasingly being considered for screening water soluble chemicals for endocrine activity. Currently, this possibly sensitive time point for thyroid disruption is not captured in the OECD TGs (detailed in Table 2). However, several promising embryo test methods for thyroid system are being developed, including a new Xenopus test guideline, 'Xenopus Embryonic Thyroid Signalling Assay' (GFP-Xenopus Embryo, Project 2.39), which is on OECD Work plan for Test Guidelines and the 'Zebrafish Eleutheroembryo Thyroid Assay' (Thienpont et al. 2011; Raldua et al. 2012)

which was recently included in an OECD scoping document (OECD series on testing and assessment no. 207, 2014). The Zebrafish Eleutheroembryo Thyroid Assay focuses on T4 concentration in the follicle cells of 5-day post fertilisation (dpf) embryos using whole mount immunofluorescence analysis. It was suggested in the OECD scoping document that additional endpoints such a quantifying mRNA levels of certain genes (PCR) or proteins (ELISA) would provide a fuller picture of disruption to the developing thyroid system (OECD series on testing and assessment no. 207, 2014). These molecular and protein approaches have been used by several authors to help elucidate specific targets of thyroid disrupting chemicals e.g. (Jia et al. 2016; Liang et al. 2015; Tu et al. 2016).

Specific neurological or behavioural end points are lacking from any of the non-mammalian TG. There is an increasing volume of fundamental research using zebrafish as a model for neurological development (Wyatt et al. 2015), therefore there are a growing number of possible assays that could be developed for regulatory testing using this species. Similar methods to the 'Zebrafish Eleutheroembryo Thyroid Assay', have recently been developed to examine thyroid hormone deiodinase expression in embryonic and larval zebrafish (Dong et al. 2013). Using 6-OH-BDE-47 to test the model Dong *et al.* found increases in mean intensity of Dio1 and Dio3 expression in the periventricular zone of brain and pronephric duct, respectively. This visual *in vivo* model highlights how compounds can have complex localised responses, which could affect neurodevelopment (Dong et al. 2013).

Zebrafish larvae high throughput assays have also recently been developed for neurological research and pharmaceutical testing (Pelkowski et al. 2011; Richendrfer & Creton 2013; Clift et al. 2014; Clift et al. 2015). These methods have shown promise to measure behavioural effects in zebrafish larvae exposed to environmental chemicals (Lovato et al. 2016).

Compared to the standard rodent model, the test methods for non-mammalian species have less integration of multiple endocrine endpoints to be assessed in one test organism. With the recent inclusion of TG 241 (LAGDA) some developmental masculinising or feminising endpoints are considered along with thyroid disruption. However, none of the Xenopus tests assess parental exposure on offspring, which considering the importance of maternal transfer of hormones and nutrients in early development in egg laying species e.g. (Brown et al. 2014) might be considered a 'gap' in testing. Interestingly, work with zebrafish has demonstrated maternal TH is essential for neural development (Campinho et al. 2014). The addition of thyroid specific endpoints into the Medaka extended one generation test (TG 240) could be a possibility to address this gap (in fish) if it was considered a high enough priority. There is also a 'Zebrafish Extended One Generation Reproduction Test' (ZEOGRT) under test guideline development (Project 2.59), although it is not known if thyroid disruption endpoints have been proposed in this test.

There are a growing number of transgenic models designed to investigate the endocrine system of fish which might be of use in testing of endocrine active (including thyroid active) chemicals. Their application for ecotoxicology testing has recently been reviewed by Lee *et al*. (Lee et al. 2015). For example, for specific thyroid endpoints, zebrafish with fluorescent proteins under the control of TSHβ (Ji et al. 2012) and thyroglobulin (Fetter et al. 2015) have been developed.

In addition, although the Avian reproduction test covers many of the important life stages where the thyroid system may be disrupted no specific thyroid measures (T4, TSH, thyroid histopathology, etc.) are assessed. An 'Avian 2-Generation Reproductive Toxicity Assay' had been tabled as a new guideline under development (Project 2.4). However, it has been removed from the most recent (July 2016) OECD Test Guidelines Programme (TGP) work plan. In non-standardised avian tests, chemicals such as perchlorate, have been shown to induce hypothyroidism (thyroid gland hypertrophy and lower thyroid hormone storage) alongside decreased embryo development and delayed hatching in maternally exposed Japanese quail chicks (Chen et al. 2008). Importantly, the US EPA published its 'Avian Two-generation Toxicity Test in the Japanese Quail'

(OCSPP 890.2100) in 2015. This EPA guideline includes thyroid specific endpoints (TH and thyroid histopathology) in Adult (F0, F1), chicks (F2) and embryos (F1).

6.4.7.4 Future testing strategies

In addition to the 'gaps' in *in vivo* testing highlighted above, it is important to bring thyroid testing in line with other endocrine disruption assays. For instance, compared to androgenic or estrogenic assays there are no validated OECD *in vitro* TG for thyroid activity. A whole host of *in vitro* assays are needed to include thyroid hormone production, biotransformation, transport, clearance, and receptor mediated responses to fully capture possible disruption.

A key issue within the thyroid disruption field is the difficulty in linking a thyroid mechanism of action to an adverse outcome. Therefore, a complementary approach is needed to link *in vitro* and *in vivo* endpoints, to gain a better understanding of which adverse effects are related to which target(s). Until such an approach is developed, it seems difficult to rely entirely on batteries of *in vitro* assays for the identification of thyroid disrupting chemicals.

One step toward in mapping these pathways out is using the Adverse Outcome Pathway (AOP) approach. For both mammals and non-mammals (amphibian and fish) there are a number of AOPs under development (AOPwiki). Of the ten non-mammal AOPs, six have amphibian metamorphosis (or disruption to) as their adverse outcome (AOP 175, 176, 188, 189, 190 and 191). These AOPs have inhibition of thyroperoxidease (TPO), Sodium Iodide Symporter (NIS), iodothyronine deiodinase 1, 2 and 3 (ID1, ID2, ID3) or Iodotyrosine deiodinase (IYD) as their Molecular Initiating Event (MIE). Another four relate to reduced young of year survival via (disruption to) anterior or posterior swim-bladder inflation in fish (AOP 155-158).These have MIE relating to ID1 or ID2 e.g. AOP 155 'Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation'.

Eight mammalian AOPs are also under development. In two of these the adverse outcome is developmental neurotoxicity in humans, with inhibition of the Sodium Iodide Symporter (NIS) (AOP 54) and interference with transthyretin (TTR) binding (AOP 152), as the MIEs. Adverse neurodevelopmental outcome in mammals is also the adverse outcome in three AOPs being developed in the US EPA. Here increased thyroid hormone catabolism via activation of hepatic nuclear receptors (AOP 8), inhibition of thyroid peroxidase (TPO) (AOP 42) and inhibition of NIS (AOP 134) are the MIEs. The last two AOPs include the adverse outcome of follicular cell adenomas and carcinomas in rats and mice, and link this to the MIE of inhibition of NIS (AOP 110) and TPO inhibition (AOP 119).

Once these conceptual pathways have been built, the next challenge will be to network them, as we well know it is unlikely that MIE occur alone, and to consider at which point these net effects breach the tipping point to initiate adversity.

Another issue is whether additional non-mammalian test models are needed. In a review paper by Pickford, the following conclusion can be found: "… Given our responsibility to reduce, refine, and replace the use of animals in toxicity testing and the variety of in vivo and in vitro approaches available, use of an amphibian metamorphosis assay alongside well-validated rodent assays in a screening battery might ultimately represent an unnecessary and unjustifiable redundancy. …" (Pickford 2010). On the other hand, the findings by Wegner et al. (2016) suggest that there is added value in non-mammalian assays.

"… the movement of toxicology and hazard assessment toward a pathway-based paradigm opens numerous opportunities in applying non-traditional approaches for hazard screening and understanding the risks of chemical exposures. Alternative species can provide valuable and relevant information more rapidly, and at lower cost, than traditional species used for human chemical risk assessment. In a pathway centric world,

all species can provide information to protect other species, each with different advantages in use, sensitivity, and accessibility. Given this perspective, the distinctions between the disciplines of human health and ecotoxicology are blurring and heading towards a more unified and integrated application of toxicological data." (Perkins et al. 2013)

Therefore, the question of developing more OECD tests for thyroid disruption perhaps comes down to the question(s) we are interested in answering, i.e. are we interested in tests that support the regulatory status-quo or do we want to develop robust test data sets to feed into future non *in vivo* methods and programs?

6.4.7.5 References

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Annex 1

OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals (as revised in 2012) as included in the OECD Guidance Document No. 150 (Annex 1.4)

Mammalian and non-mammalian Toxicology	
Level 1 Existing Data	• Physical & chemical properties, e.g., MW reactivity, volatility, biodegradability • All available (eco)toxicological data from standardized or non-

and Non-Test Information	standardized tests. • Read across, chemical categories, QSARs and other *in silico* predictions, and ADME model predictions

Level 2 *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non-mammalian methods)	• Oestrogen or androgen receptor binding affinity • Oestrogen receptor transactivation (OECD TG 455 – OECD TG 457) • Androgen or thyroid transactivation (If/when TGs are available) • Steroidogenesis in vitro (OECD TG 456) • MCF-7 cell proliferation assays (ER ant/agonist) • Other assays as appropriate

	Mammalian Toxicology	**Non-Mammalian Toxicology**
Level 3 *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1]	• Uterotrophic assay (OECD TG 440) • Hershberger assay (OECD TG 441)	• Xenopus embryo thyroid signalling assay (When/if TG is available) • Amphibian metamorphosis assay (OECD TG 231) • Fish Reproductive Screening Assay (OECD TG 229) • Fish Screening Assay (OECD TG 230) • Androgenized female stickleback screen (GD 140)
Level 4 *In vivo* assays providing data on adverse effects on endocrine relevant endpoints [2]	• Repeated dose 28-day study (OECD TG 407) • Repeated dose 90-day study (OECD TG 408) • 1-generation reproduction toxicity study (OECD TG 415) • Male pubertal assay (see GD 150, Chapter C4.3)[3] • Female pubertal assay (see GD 150, Chapter C4.4)[3] • Intact adult male endocrine screening assay (see GD 150, Chapter Annex 2.5) • Prenatal developmental toxicity study (OECD TG 414) • Chronic toxicity and carcinogenicity studies (OECD TG 451-3) • Reproductive screening test (OECD TG 421 if enhanced)	• Fish sexual development test (OECD TG 234) • Fish Reproduction Partial Lifecycle Test (when/If TG is Available) • Larval Amphibian Growth & Development Assay (when TG is available) • Avian Reproduction Assay (OECD TG 206) • Mollusc Partial Lifecycle Assays (when TG is available) [4] • Chironomid Toxicity Test (TG 218-219) [4] • Daphnia Reproduction Test (with male induction) (OECD TG 211) [4] • Earthworm Reproduction Test (OECD TG 222) [4] • Enchytraeid Reproduction Test (OECD TG 220) [4] • Sediment Water Lumbriculus

	• Combined 28-day/reproductive screening assay (OECD TG 422 if enhanced) • Developmental neurotoxicity (OECD TG 426)	Toxicity Test Using Spiked Sediment (OECD TG 225) [4] • Predatory mite reproduction test in soil (OECD TG 226) [4] • Collembolan Reproduction Test in Soil (TG OECD 232) [4]
Level 5 *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	• Extended one-generation reproductive toxicity study (OECD TG 443)[5] • 2-Generation reproduction toxicity study (OECD TG 416 most recent update)	• FLCTT (Fish LifeCycle Toxicity Test) (when TG is available) • Medaka Multigeneration Test (MMGT) (when TG is available) • Avian 2 generation reproductive toxicity assay (when TG is available) • Mysid Life Cycle Toxicity Test (when TG is available)[4] • Copepod Reproduction and Development Test (when TG is available)[4] • Sediment Water Chironomid Life Cycle Toxicity Test (OECD TG 233) [4] • Mollusc Full Lifecycle Assays (when TG is available) [4] • Daphnia Multigeneration Assay (if TG is available) [4]

Footnote and notes to the OECD Revised Conceptual Framework (Figure 1)

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-ED mechanisms.

[3] Depending on the guideline/protocol used, the fact that a substance may interact with a hormone system in these assays does not necessarily mean that when the substance is used it will cause adverse effects in humans or ecological systems.

[4] At present, the available invertebrate assays solely involve apical endpoints which are able to respond to some endocrine disrupters and some non-EDs. Those in Level 4 are partial lifecycle tests, while those in Level 5 are full- or multiple lifecycle tests.

[5] The Extended one-generation reproductive Toxicity Study (OECD TG 443) is preferable for detecting endocrine disruption because it provides an evaluation of a number of endocrine endpoints in the juvenile and adult F1, which are not included in the 2-generation study (OECD TG 416) adopted in 2001.

Note 1: Entering at all levels and exiting at all levels is possible and depends upon the nature of existing information and needs for testing and assessment.

Note 2: The assessment of each chemical should be made on a case by case basis, taking into account all available information.

Note 3: The framework should not be considered as all inclusive at the present time. At levels 2, 3, 4 and 5 it includes assays that are either available or for which validation is under way. With respect to the latter, these are provisionally included.

7. BACKGROUND AND TOPIC OUTLINES FOR THE WORKSHOP DISCUSSION GROUPS

Briefing papers were prepared to structure the deliberations of the three Discussion Groups. These papers outlined the background to the three topics and made suggestions for discussion topics.

7.1 BACKGROUND AND TOPIC OUTLINE FOR DISCUSSION GROUPS 1 A, B: ASSAYS AND ENDPOINTS FOR THYROID DISRUPTION IN MAMMALIAN ORGANISMS – STATUS QUO AND PERSPECTIVES FOR ENHANCEMENTS

Background

Material supporting the discussions of this group can be found in the following background documents:

Information on the basics of the thyroid system and on pathways that induce thyroid hormone alterations are in **Background Paper 1 <BG1 adverse effects human>** which describes evidence from epidemiological and clinical studies.

A description of current OECD guidelines, together with perspectives on some enhancements that could be used to address thyroid disrupting modes of action can be found in **Background Paper 4 <BG4 current test methods gaps>**. Key points from this material are:

- *In vitro* assays relevant to thyroid disruption are currently not part of testing guidelines.
- In mammalian assays of level 3 of the OECD framework, thyroid-related endpoints are not mandatory. In levels 4 and 5, there are very few test guidelines that include both, measurements of thyroid hormone levels and thyroid histopathology.
- No current guideline study addresses the issue that correct levels of thyroid hormone may not reach the developing brain, which could consequently cause developmental neurotoxicity.
- Endpoints and assays informative of thyroid hormone downstream effects (e.g. taking account of non-uniform distribution of brain cells sensitive to TH, hippocampal gene expression, alteration in hippocampal anatomy and function, etc are not implemented
- The importance of timing of exposure to thyroid disrupting chemicals is insufficiently covered.
- The impact of thyroid disruption on cardiovascular disease and lipid metabolism is currently a blind spot in test systems relevant to thyroid disruption.

Background Paper 4 <BG4 current test methods gaps> also maps out possibilities of including endpoints and assays that may fill the gaps listed above:

- Inclusion of measures of down-stream effects on the brain, incorporating information about neuronal migration and proliferation, oligodendrocyte differentiation and cerebellar histogenesis
- Inclusion of endpoints relevant to the effects of thyroid disruption on the cardiovascular system and on lipid metabolism

Important aspects of the above issues are discussed in the **case studies** of fipronil, PBDE and mancozeb (see section 5).

Suggested reading of background documents in preparation of GD 1 ab

Information useful for this Discussion Group may be found in the following background documents:

- **Background paper 1 adverse effects human**
- **Background paper 4 current test methods gaps**
- **Case study fipronil**
- **Case study PBDE/perfluorinated compounds/PCB**
- **Case study mancozeb**

Suggested discussion topics and questions

1. Which *in vitro* and *in vivo* endpoints can be regarded as indicating a thyroid mode of action?
2. Which non-guideline assays or studies and new endpoints, and which enhancements to current internationally accepted guideline assays/studies could be used to address most adequately thyroid disrupting modes of action and/or adverse outcomes?
3. How could these new endpoints or assays be included in new test strategy developments?
4. In the context of identification of thyroid disrupting substances, should the adverse effects of thyroid hormone under- or over-activity on the cardiovascular system and on lipid metabolism documented in the human be considered? If so, how?
5. Can a combination of *in vitro* assays support judgements of adversity with relevance to the human, and can this replace studies in whole animals?

7.2 BACKGROUND AND TOPIC OUTLINE FOR DISCUSSION GROUPS 2 A, B: THE HUMAN RELEVANCE OF MODELS OF PATHWAYS OF THYROID DISRUPTION

Background

A description of pathways relevant to thyroid disruption and particularly thyroid hormone insufficiency can be found in **Background paper 1** (section 6). This includes inhibition of iodine uptake (NIS), inhibition of thyroid hormone synthesis (TPO inhibition), upregulation of hepatic metabolism relevant to the clearance of thyroid hormones, displacement of thyroid hormones from serum distributor proteins and/or cellular transmembrane transporter proteins for TH.

Human clinical and epidemiological studies show that maternal subclinical hypothyroidism, characterised by normal thyroid stimulating hormone (TSH) with thyroid hormone (TH) levels towards the lower end of the population distribution, is associated with compromised cognitive development in their children (IQ loss). Recent studies also suggest associations with autism spectrum disorder, attention deficit hyperactivity disorder and schizophrenia.

Rises in the serum levels of TSH within the normal range are associated with increased risks of thyroid cancer, the fastest rising cancer among women and men (see case study on thyroid cancers in section 5).

Several studies among humans have uncovered associations between exposure to pollutants and TH patterns typical of subclinical hypothyroidism. This is the case with combined exposures to perchlorate and thiocyanate, when accompanied by iodine deficiency. Similar associations have been shown with mercury, certain arsenic species, certain organochlorine pesticides, poly-aromatic hydrocarbons and perfluorinated compounds (see **Background Paper 1** in section 6 and the case studies in section 5).

Decreases in TH levels as a result of administration of chemical substances have been seen in numerous rodent studies (for further detail see **case studies** on fipronil and perchlorate, section 5). The relevance of these observations for human risk assessment is often questioned with reference to quantitative differences of the rat thyroid system relative to the adult human system (see **case studies** on thyroid cancer and perchlorate, section 5).

However, new evidence suggests that the capacity of the thyroid system to compensate for the effects of chemicals leading to thyroid insufficiency depends on the life stage, both in humans and rats (summarized in **Background Papers 1 and 3**). Newborn babies do not have the reserves of the adult thyroid system and are therefore more sensitive to exposures to thyroid disruptors (**Background Papers 1 and 3**).

Furthermore, decreases of TH in rodents are often not judged to be adverse, in the absence of other effects (e.g. thyroid histopathology etc.) (**case study** on fipronil).

The debate about evaluations of observations in the rat is important when it comes to the identification of substances as thyroid carcinogens and their relevance to human risk assessment. Many rodent thyroid carcinogens lead to increased TSH levels, mainly in male rats, often through a mechanism involving increased TH clearance through upregulation of hepatic conjugation reactions. A rise in TSH is seen as the key event of thyroid carcinogenesis (see **case study** on thyroid cancers). USEPA and IARC guidance regarding the identification of thyroid carcinogens rests on the presumption that rodent thyroid carcinogens may pose a cancer hazard to humans, and that in the absence of chemical-specific data humans and rats should be assumed to be equally sensitive.

Nevertheless, the relevance of this pathway for human risk assessment, and for identifying substances as thyroid disruptors is often called into question. In the recent European Commission Joint Research Centre (JRC) screening methodology for identification of endocrine disruptors in the context of an impact assessment (for more detail see **case study** on fipronil) this point is summarised as follows:

Histopathological findings in rat thyroid and increased thyroid weight in presence of liver histopathology (including liver enzyme induction) were attributed to a liver-mediated mechanism not considered to be ED-mediated. Since in the frame of this screening methodology enhancement of the metabolism and excretion of thyroid hormones by the liver was not considered as an endocrine MoA, such effects were not considered relevant to conclude on ED.

However, from an endocrinological viewpoint, both hormone synthesising and removing pathways will be viewed as important in evaluating impacts on thyroid hormone action, including increased clearance by hepatic metabolism (see **case study** on thyroid cancers).

Suggested reading of background documents in preparation of DG 2 ab

Information useful for this Discussion Group may be found in the following background documents:

- **Background paper 1 adverse effects human**
- **Background paper 3 interpretation of data**
- **Case study fipronil**
- **Case study TSH thyroid cancer**

Suggested discussion topics and questions

1. In the context of thyroid disruption and the identification of thyroid disrupting substances: Which *in vivo* endpoints in experimental studies should be regarded as adverse?

2. Should the induction of thyroid hormone insufficiency through increased hepatic conjugation reactions be judged as thyroid disruption?
3. What is the human relevance of different mechanisms for thyroid disrupting effects?
4. Should the induction of thyroid tumours in rodents be taken to designate a substance as a presumed human carcinogen? Are USEPA and IARC guidelines which presume that rodent thyroid carcinogens may pose a cancer hazard to humans still valid? In the absence of chemical-specific data, should humans and rats be assumed to be equally sensitive?
5. Should the induction of thyroid tumours in rodents be taken to identify a substance as a thyroid disruptor and/or a carcinogen?
6. Should the human relevance of thyroid disrupting effects in animals be judged in terms of species (e.g. "the rat is not relevant") or should these judgements be made in relation to specific pathways and mechanisms of thyroid disruption?

7.3 BACKGROUND AND TOPIC OUTLINE FOR DISCUSSION GROUPS 3 A, B : THYROID SYSTEMS ACROSS TAXA: THE RELEVANCE OF MAMMALIAN DATA IN ENVIRONMENTAL ASSESSMENTS AND OF NON-MAMMALIAN DATA IN HUMAN HEALTH ASSESSMENT AND POSSIBILITIES OF ENHANCING TEST GUIDELINES ACCORDINGLY

Background

The basic features of the thyroid system are conserved across all taxa, although there are differences in detail (see **Background Paper 2**, section 6).

A description of endpoints and test organisms relevant to non-mammalian species currently used in OECD test guidelines can be found in **Background Paper 4**, section 6.

An issue for debate is the extent to which the outcome of non-mammalian assays can inform the hazard identification of thyroid disruptors in the human toxicology arena.

Conversely, to what degree are mammalian thyroid disruptor assays informative in terms of anticipating the impact on ecosystems?

Relevant to this debate are the gaps in current OECD test guidelines for thyroid disruption in non-mammalian tests. **Background Paper 4** has summarised these gaps as follows:

- In test guidelines for non-mammalian species, there are no measurements of thyroid hormone levels or TSH which complicates evaluations in relation to mammalian assays
- There is little integration of multiple endocrine endpoints in non-mammalian tests
- No set neurological or behavioural end points are established in non-mammalian assays
- The OECD conceptual framework does not incorporate embryo culture techniques
- The maternal transfer of chemicals to offspring is not covered in non-mammalian tests
- There are currently no avian models relevant for thyroid disruption

Background Paper 4 makes specific suggestions for dealing with these gaps, and proposes to consider new, sensitive non-mammalian assays for the identification of thyroid disruptors. These are also considered in the **case study** of perchlorate, section 5.

Suggested reading of background documents in preparation of GD 3 ab

Information useful for this Discussion Group may be found in the following background documents:

- **Background paper 4 current test methods gaps**
- **Background paper 2 thyroid disruption across taxa**
- **Case study perchlorate**

Suggested discussion topics and questions

1. In which respect can data from rodent studies be used to inform about effects on rodents/mammals in the environment?
2. In which respect can data from amphibian or fish studies be used to inform about possible effects in rodents/mammals?
3. When and how during testing should we take into consideration that the thyroid hormone system is highly conserved between taxa?
4. Should current testing guidelines be enhanced by adding additional endpoints and assays?

8. WORKSHOP REPORT

This section describes the proceedings of the workshop on thyroid disruption held on 29-31 March 2017 at ANSES in Maisons-Alfort, France.

The workshop concept, its objectives and its agenda can be found in sections 3 and 4 of this report.

The workshop was conducted on the basis of extensive material distributed to all participants in advance. This material consisted of:

- Case studies (section 5)
- Background papers (section 6), and
- Background and topic outlines for the workshop discussion groups (section 7).

8.1 Workshop participants

Invited workshop participants were from EU Member State competent authorities, competent authorities from the USA, Canada and Japan, industry, academia and non-governmental organisations. All members of the Scientific Expert Group and the Steering Group (section 2) also attended.

A list of participants can be found in Annex 1 of this report.

8.2 Workshop report: Summary of formal presentations

In this section, a brief summary of formal workshop presentations is given. The presentations are available in Annex 2 to this report.

Peter Korytar (DG ENV, European Commission) and Roger Genet (Director General of ANSES) welcomed the participants and Andreas Kortenkamp (Brunel University London, UK) introduced the concept of the workshop.

Tom Zoeller (University of Massachussetts, Amherst, USA) delivered the keynote lecture of the workshop, co-authored by Barbara Demeneix, Josef Koehrle, and Catherine Viguie: *"Thyroid disruptor research in the 21st century"*. He developed what can be referred to as the "idealized view" of the thyroid system, where decreases in circulating thyroid hormones are compensated by increases in TSH which in a feedback loop restore serum thyroid hormones to their original levels. However, this view is at odds with observations from experimental studies in which decreases in T4 following chemical exposures were not always accompanied by decreases in T3 or increasing serum levels of TSH. Specifically referring to a paper by Klaassen and Hood (Tox Pathol 29, 34) he emphasized examples where T4 reductions left TSH concentrations entirely unchanged, depending on the chemicals used to induce T4 decreases. There are even examples where continued suppression of T4 after exposure to certain chemicals resulted in decreases of serum TSH. Thus, the "idealized view" cannot explain observations of chemically induced alterations of the thyroid system. This may render the data sets of serum thyroid hormone measurements contained in many chemical dossiers difficult to interpret. He further presented evidence that altered thyroid hormone serum concentrations can be associated with adverse effects even though the hormone levels remain within the population reference range. The traditional reliance on the diagnostic value of serum thyroid hormone concentrations is further complicated by new evidence of autonomous regulation of thyroid hormone action at the tissue level, without involvement of the HPT axis and corresponding changes in serum thyroid hormones. The inevitable conclusion is that altered TSH levels are not the "be-all and end-all" marker of thyroid hormone action. Another example illustrating this point is the Allan-Herndon-Dudley Syndrome which is caused by mutations in the thyroid hormone transporter MCT8. This results in low serum T4, elevated serum T3 and normal to elevated TSH, a clear case where low T4, together with normal to slightly elevated TSH, leads to adverse outcomes in male humans. This disease cannot currently be treated. He also discussed examples

that challenge the received opinion about the compensatory capacity of the thyroid system. For example, PTU exposure leads to reductions in circulating T4, which the brain counteracts by maintaining T3 tissue levels through increased activity of deiodinases which convert the remaining T4 stores to T3. However, despite the resulting relatively stable T3 brain tissue levels, the expression of a neuronal specific gene representative of intracellular T3 action (RC3) decreases. The importance of serum thyroid hormones also has to be interpreted carefully in the context of equilibria formed with distributor proteins, such as thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin.

Patience Browne (OECD, France) gave an overview of the existing test guidelines for thyroid disruption. Although some of the OECD Conceptual Framework Level 4-5 studies incorporate thyroid endpoints, there are currently no validated Level 2 *in vitro* mechanistic assays, and limited Level 3 *in vivo* studies to support the evaluation of thyroid disrupting effects.

Alice Baynes (Brunel University London, UK) presented new insights from ecotoxicological animal models (fish, amphibians). Despite some differences in physiology and anatomy, there is significant conservation of the basic make-up of the thyroid hormone system across taxa. The current battery of tests for thyroid disruption in wildlife is strongly based on amphibians, with a neglect of other taxa. The presentation was based on Background paper 2 (section 6.2).

Eva Fetter (Umweltbundesamt, Germany) gave an overview of substances interacting with thyroid hormone synthesis and their ability to trigger adverse effects on the HPT and HPG axes from an ecotoxicological perspective. She illustrated important principles with a case study of perchlorate.

Marta Axelstad (Technical University of Denmark, Denmark) presented a summary of the fipronil case study which dealt with the relevance of the rat as a model for humans in the context of thyroid hormone insufficiency caused by induction of hepatic conjugating enzyme systems (see section 5. 1).

Niklas Andersson (ECHA, Finland) discussed the approaches employed by the European Chemicals Agency (ECHA) in its evaluations of thyroid disrupting chemicals, especially in interpreting the relevance of thyroid hormone insufficiency produced *via* induction of hepatic conjugating enzyme systems. ECHA's requests for information from data submitters have to be proportional and tailored to information needs. However, this is currently problematic due to a lack of validated *in vitro* tests indicative of thyroid disruption and the gaps in *in vivo* tests regarding thyroid-relevant endpoints.

Olwenn Martin (Brunel University London, UK) and Marta Axelstad (Technical University of Denmark, Denmark) jointly presented the results of the PBDE/perfluorinated compounds/PCB and mancozeb case studies. These case studies discussed the consequences of down-stream effects of maternal and early post-natal thyroid hormone insufficiency and other mechanistic aspects of thyroid disruption (see sections 5.4 and 5.2, respectively).

This was followed by presentations from Andreas Kortenkamp (Brunel University London, UK) in which he summarized the case study on the role of TSH in thyroid cancer (section 5.5) and the evidence of associations between chemical exposures and thyroid disruption from human epidemiological studies (section 6.1).

8.3 Workshop report : Deliberations of the discussion groups

Workshop participants were split into six groups, to discuss three topics:

- Assays and endpoints for thyroid disruption - *status quo* and perspectives for enhancements
- The human relevance of models of thyroid disruption pathways, and
- Thyroid systems across taxa: the relevance of mammalian data in environmental assessments and of non-mammalian data in human health assessments.

There were two discussion groups for each of the topics. A consolidated summary of the deliberations of each of these 3 pairs is presented below, without always differentiating between the parallel-running pairs of discussion groups.

8.3.1 Discussion groups 1 a b : Assays and endpoints for thyroid disruption – status quo *and perspective for enhancements*

The deliberations of groups 1a and 1b were based on the presentations at the workshop, the workshop materials and especially the document 'Background and topic outline for Discussion Groups 1 a, b' (given in section 7.1). The suggested discussion topics and questions in this document were:

1. Can a combination of *in vitro* assays support judgements of adversity with relevance to the human, and can this replace studies in whole animals?
2. Which non-guideline assays or studies and new endpoints, and which enhancements to current internationally accepted guideline assays/studies could be used to address most adequately thyroid disrupting modes of action and/or adverse outcomes?
3. How could these new endpoints or assays be included in new test strategy developments?
4. In the context of identification of thyroid disrupting substances, should the adverse effects of thyroid hormone under- or over-activity on the cardiovascular system and on lipid metabolism documented in the human be considered? If so, how?
5. Which *in vitro* and *in vivo* endpoints can be regarded as indicating a thyroid mode of action?

Decreases in serum thyroid hormones – an adverse effect?

Both groups discussed whether decreases in thyroid hormones (mainly T4) seen as a result of chemical exposures in animal studies should in themselves be presumed as adverse. These discussions were motivated by the recognised relationships between low T4 and effects on the developing brain in humans.

While the question was not answered conclusively, there was agreement that reductions in T4 levels should act as a trigger for further studies of F1 generations, for example as part of TG 421/422, 426, 443, depending on the other information requirements.

To some participants it was clear that regulatory decisions based on findings of diminished serum T4 would protect against downstream effects (*e.g*. developmental neurotoxicity) in humans. These participants agreed that changes in T4 in animal studies are predictive of adverse consequences on the developing human brain and should therefore be regarded as sufficient for making regulatory decisions.

Some participants expressed the view that decreased serum T4 occurring *via* hepatic enzyme induction in rodent studies should be considered relevant in the context of regulatory decisions unless, on the basis of further compound specific information, it would be possible to exclude the relevance of such effects. In the context of human risk assessments, this could for instance be tested *in vitro* using primary human hepatocytes. Discussion group 1b only briefly discussed this and no clear conclusion was formulated. However, several experts in the group expressed the view that effects on T4 are signs of endocrine effects, irrespective of whether these result from increased breakdown and excretion (due to hepatic enzyme induction) or decreased synthesis.

There was concern that current guideline studies are insufficiently sensitive for the reliable detection of developmental neurotoxicity, even when T4 levels are decreased. This lack of suitable and sensitive endpoints makes it problematic to establish potential developmental neurotoxicity and to negate possible concerns that arise from T4 effects even when developmental neurotoxicity is not observed.

The relevance of *in vitro* assays in making judgements about adverse thyroid disrupting effects

Discussion group 1b specifically addressed this question (question 1 in the background and topic paper). Participants concluded that observations of effects in a number of *in vitro* assays can provide important Mode of Action (MOA) information which can support interpretations of the relevance of adverse effects observed in a model animal species to humans. As such, the outcome of *in vitro* tests is useful for setting priorities for subjecting substances to further testing. However, negative *in vitro* results were considered not to be conclusive due to the many MOAs that may lead to thyroid disruption and the limited metabolising capacities of *in vitro* tests. It was concluded that combinations of *in vitro* assays can presently not replace studies in whole animals.

Participants developed different proposals as described below for the better use of endpoints in existing guidelines and saw the need for further guidance in the interpretation of thyroid relevant endpoints in existing guidelines in the context of AOPs/MOA frameworks.

Perspectives for improvements of current guideline studies to incorporate thyroid disrupting effects

Both discussions groups made several recommendations on how to improve the existing testing for thyroid disruption. The suggestions were sub-divided as follows:-

- addition of "standard endpoints", *i.e*. endpoints that are already routinely measured in other TGs,
- addition of "new endpoints" to existing test guidelines, *i.e*. endpoints that are presently not included in any guidelines and
- identification of new areas of research, or new assay development that need to be performed in order to improve investigation of thyroid disruption in the longer term.

The following sections discuss each of these suggestions in turn.

Addition of standard endpoints to existing guideline studies

In discussing the addition of "standard endpoints", discussion group 1a considered, without reaching a consensus, making the developmental neurotoxicity cohort of TG 443 mandatory. Group 1b also dealt with this issue and recommended that the DNT cohort of TG 443 should be mandatory if there is a thyroid concern. This group also recommended that TG 443 (but not TG 416) should be mandatory for all substances.

All participants recommended to make measurements of T4 and TSH mandatory in TG 407 and 408. TH and TSH measurements need to be performed with methods validated for the species used to test for thyroid axis interference. An enhancement of TG 407 (28 days study) in particular was seen as both relevant and feasible within a short period of time by changing the status of thyroid hormone measurements from optional to mandatory, as was done in the recently updated TG 422 (combined 28 day and reproductive toxicity screen). Significant T4 reductions should trigger further testing in the F1 generation.

Group 1a recommended that T4 changes should also be measured in TG 241 (LAGDA).

If exposure to a test chemical is primarily *via* the dermal or inhalative route, or if no mammalian data are available, T4 decreases observed in TG 241 (amphibian) were also seen as a trigger for mammalian studies with inclusion of an F1 generation. This recommendation was motivated by the conservation of the thyroid system between taxa, as succinctly stated in a recent publication: "frogs are little humans that hop" (Sachs and Buchholz 2016). Exceptions to this would be in cases where frog metabolism of toxicants following exposure *via* the dermal route is known to be different from mammals.

Inclusion of new endpoints into existing test guidelines

Specific investigations of brain morphology in developmentally exposed animals were seen by all participants as a very relevant addition to existing test guidelines. In the F1 generation of TG 421/422, 426 and 443, both discussion groups found it important to include sensitive and specific markers of thyroid hormone action in the brain. This could probably be obtained by evaluation of heterotopias and oligodendrocyte differentiation in brain tissue from perinatally exposed offspring. Cortical heterotopias are indicative of gestational T4 deficits in rodent pups, whereas changes in oligodendrocyte differentiation are indicative of early postnatal T4 deficits. Both endpoints can be measured in PND 13 and 22 pups.

With a view to incorporating thyroid-related effects that go beyond developmental neurotoxicity and to include possible effects on the cardiovascular system and on lipid metabolism, participants suggested the addition of the following new endpoints to existing test guidelines:

- Comprehensive lipid profiles (cholesterol, TAG, HDL/LDL)
- Heart histology and heart weight, body temperature, body weight (increase, decrease) should be considered
- Potentially: heart rate and blood pressure, atherosclerosis (in chronic studies), although the difficulties of measuring these, and the relative resistance of rats to artheriosclerosis, were acknowledged
- Improvements in assessing thyroid histology by adopting guidance from the American Medical Association (Bianco et al. 2014), and GD 82 for AMA histopathology
- Thyroid histology guidance amenable to automation (Hooth and Wolf 1998 Tox Path)

The following changes to study design and study methods could also be implemented:

- Methods alternative to enzyme immunoassays should be considered for measuring thyroid hormones (and their metabolites)
- Regardless of method, it should be required that laboratories demonstrate proficiency in measuring thyroid hormones reliably
- Samples for hormone analysis should be collected during 9-11 am to reduce diurnal variation
- Guidance from German physicians for hormone measurements should be considered (Richtlinien der Bundesaerztekammer)
- TSH antibodies may be unreliable for measuring TSH changes in rodents, as an alternative, TSHβ transcripts could be measured in the pituitaries of rodents, amphibians, fish, which would also increase the comparability among taxa
- Serum volumes may be limiting if using commercially available kits, other methods may require smaller volumes

New areas of assay development and research

Discussion group 1b proposed the development of a new assay, an *in vivo* thyroid screen. This proposal was motivated by the fact that many substances had been studied by using

older versions of test guidelines, *e.g*. TG 407, where no endpoints for effects on thyroid hormones were included. Since it seemed disproportionate to fill this data gap by re-testing with an updated and improved version of *e.g*. TG 407, testing with an abbreviated *in vivo* thyroid screen with shorter exposure periods could accomplish filling this data gap quickly and effectively. The proposal is based on the observation that changes in thyroid hormones in adult experimental animals can often be seen already after a relatively short exposure period (4-5 days). The proposed screen could be developed based on TG 407 and/or TG 422, with a correspondingly shorter exposure period. However, thyroid effects mediated *via* hepatic enzyme induction may require a somewhat longer exposure period of *e.g*. 14 days. In the plenary session, a streamlined Intact Adult Male Endocrine Screening Assay listed in the OECD Conceptual Framework was recommended for that purpose.

Both groups recommended further development of *in vitro* tests addressing thyroid MOA, for example assays investigating the TPO, TTR, TBG, TRH, TSH, NIS, Mct8, TR-dependent transcription, and deiodinases (type 1, 2 and 3). It was recommended to carefully consider the selection of reference chemicals (for individual/all *in vitro* assays) and to begin with the OECD thyroid scoping document 207.

Both groups also discussed several new endpoints that may in the future (short or long term) provide relevant evidence for thyroid disrupting MOA and downstream effects and potentially for enhancements of existing guidelines or new guidelines. A list of these endpoints is given below:

- *In vivo* studies: measurements of TPO/NIS transcript in thyroid and TTR/TBG transcripts in liver
- *In vitro* oligodendrocyte differentiation/development (longer term)
    - Uniquely and specifically thyroid-dependent
    - Can be interpreted with recommended CNS histopathology
- *In vivo* brain heterotopia association with decreased maternal T4 needs additional investigation
- New behavioural test(s) for assessment of thyroid-related DNT (research)
- Distribution of white *vs*. black matter in brains as marker for thyroid-related effect on the developing brain (ongoing, research)
- *In vivo* retinal development/electroretinalgrams (after prolonged exposure and/or extended observation times)
- *In vivo* tissue-specific thyroid effects (*e.g*. DI activity and transcript, after prolonged exposure and/or extended observation times)
- Indications of altered thyroid hormone signalling in bone
- Gene markers/expression profiles (short term; literature search)
    - Kits already exist (qPCR in HT format)
    - Validation/literature search needed to confirm response to thyroid active compounds
    - Marker genes for brain development detailed in scientific publications
    - Marker genes for thyroid cancer
    - Marker genes for cardiovascular disease
    - Marker genes for lipid metabolism detailed in scientific publications
- Fish testing – in research phase (variable stages of development?)
    - Zebrafish embryos T3/T4 measurable from embryo day 0 (Chang et al. 2012)

- ZF brain development
- T4/TSH fish, thyroid histology in *in vivo* studies, eye development, swim bladder inflation

8.3.2 Discussion groups 2 a b : The human relevance of models of thyroid disruption pathways

The discussions in groups 2a and 2b were based on the presentations at the workshop and on the workshop materials, specifically the document 'Background and topic outline for Discussion Groups 2 a, b' (see section 7.2) distributed before the workshop. The suggested discussion topics and questions in this document were:

1. In the context of thyroid disruption and the identification of thyroid disrupting substances: Which *in vivo* endpoints in experimental studies should be regarded as adverse?
2. Should the induction of thyroid hormone insufficiency through increased hepatic conjugation reactions be judged as thyroid disruption?
3. What is the human relevance of different mechanisms for thyroid disrupting effects?
4. Should the induction of thyroid tumours in rodents be taken to designate a substance as a presumed human carcinogen? Are USEPA and IARC guidelines which presume that rodent thyroid carcinogens may pose a cancer hazard to humans still valid? In the absence of chemical-specific data, should humans and rats be assumed to be equally sensitive?
5. Should the induction of thyroid tumours in rodents be taken to identify a substance as a thyroid disruptor and/or a carcinogen?
6. Should the human relevance of thyroid disrupting effects in animals be judged in terms of species (*e.g*. "the rat is not relevant") or should these judgements be made in relation to specific pathways and mechanisms of thyroid disruption?

Adversity of thyroid-related endpoints

Dealing with question 1 above, Group 2a began by discussing thyroid histopathological changes and quickly arrived at the conclusion that changes in mammalian thyroid histopathology should be regarded as indicative of an adverse effect. However, the value of histopathological observations on their own (as required in various OECD test guidelines) can sometimes be relatively limiting, particularly as the thyroid is not the only target organ for thyroid hormone insufficiency (during development this is the brain). The interpretation of histopathological findings can be increased by accompanying measurements of thyroid hormones.

The discussion then turned to the relevance of decreases in T4. Group 2a agreed that such decreases, when observed in a reliable study, are of concern. If no further information is available, and if further data dispelling concerns are unlikely to be forthcoming, decreases in T4 should be treated on the basis of the presumption that adverse effects might materialize. The same presumption should be made in the case of increases in T4.

Group 2b approached the question of adversity of thyroid-related endpoints from a slightly different angle, by considering the WHO/IPCS definition of adversity:

"*A change in the morphology, physiology, growth, development, reproduction or lifespan of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences*."

After some debate, group 2b agreed that a lowering of serum T4 alone would not be sufficient to demonstrate adversity according to this definition. Some participants had argued that the definition, as formulated, implied a requirement for evidence of a downstream effect. Others made the point that in the clinical setting a change in serum

thyroid hormones can be sufficient to reach a diagnosis. However, whether the patient requires treatment typically depends on the presence of other symptoms such as fatigue or fluid retention.

Discussions turned to whether other measures of serum thyroid hormones could help interpretation, such as T3 or the free hormones fT3 and fT4, as well as the practical and technical difficulties encountered to reliably measure these in experimental animals. Both groups agreed that measuring free thyroid hormones appears attractive from a purely scientific perspective but pointed to several factors that make such measurements challenging or even impossible in practice. Such factors are the high cost of assay kits, their reliability, and the amount of serum available from animal studies which often is the limiting factor for reliable determinations of free T4. The most informative endpoint was suggested to be the ratio T4/T3 and this would be achievable in practice.

Group 2b agreed that in some cases, the magnitude of the change in T4 (or T4/T3, or TSH) may be sufficient to conclude that a substance has an adverse effect. However, further research was deemed necessary in order to specify and validate the range beyond which a change in thyroid hormones alone is sufficient to conclude on the adversity of the effect.

Participants of both groups agreed that, in all cases, a decrease in T4 should at the very least trigger further investigations, although it was currently not clear what those investigations should be. In making a decision on whether a decrease in T4 was a treatment-related effect, the importance of reliable data was frequently emphasised; *i.e*. sufficient numbers of animals, and the synchronisation of sample collection. Addition of a molecular read-out may be sufficient to conclude that an effect is adverse (*e.g*. existing AOP, clinical and epidemiological evidence that support strengthen the plausible link).

Additional endpoints

Group 2a discussed current test guidelines of developmental neurotoxicity in relation to thyroid disruption. It was agreed that there are issues with the sensitivity of developmental neurotoxicity endpoints. Concerns about the high likelihood of false negatives were expressed. The group recognized that these tests are insufficiently sensitive to detect the magnitude of change in T4 typically seen with environmental chemicals. Similar discussions took place in group 2b, with an orientation towards the relevance of the rat model for neurodevelopmental effects. Developmental neurotoxicity is currently considered the gold standard but it is not necessarily a sensitive enough model. Group 2b discussed several suggestions to improve this protocol. As brain development occurs also postnatally in the rat, it was proposed that the pups may be dosed directly if the substance is thought not to be transferred through lactation. Other suggestions included adding the measurement of T4 in the pups or including downstream molecular markers of TH action.

Participants of group 2b also turned their attention to tissue-specific effects. Peripheral hormone levels in blood will not predict tissue-specific effects, nor is it possible to extrapolate between organs. Tissue-specific readouts are therefore necessary.

Several additional endpoints that would be informative in this respect were suggested. T4/T3 can be measured specifically in the liver, heart, brain, bone and adipose tissue.

Hepatic deiodinase 1 (D1) was considered an important liver marker of TH status

For effects on the heart, change in the heart weight is a sensitive if non-specific endpoint for hyperthyroidism and is already included in TG 407 (28 days). However it is not currently interpreted as a thyroid sensitive endpoint.

In the pituitary, TSH mRNA levels can be indicative of hypothyroidism. Useful endpoints in the brain include the RC3/Neurogranin gene.

The functional significance of specific thyroid hormone transporting proteins was also discussed. It was noted that the human relevance of rodent models is often challenged with reference to differences in TBG levels relative to humans (rats have lower TBG). However, it was pointed out that the TTR-bound fraction is the most relevant one in terms of determining tissue availability of thyroid hormones, and that the TBG-bound fraction is less relevant in this respect. Therefore, the relevance of the rat model should not be dismissed on the grounds of low TBG levels in adult rats.

Cholesterol biosynthesis, testis weight or the ovarian cycle can all be affected by disruption of thyroid hormone homeostasis. A general comment is that the endpoints above are typically sensitive but not specific to thyroid hormone disruption. They will therefore need to be considered in context with other endpoints. Of those that are already included in validated tests, they tend not to be interpreted in the light of thyroid disruption.

Thyroid hormone insufficiency through induction of hepatic conjugation reactions

Group 2a dealt with thyroid hormone insufficiencies that occur by induction of hepatic conjugation reactions in the wider context of other modes of action that also bring about thyroid hormone reductions, *e.g. via* inhibition of NIS or TPO. There was a consensus that the latter two modalities, if observed in the rat, should be deemed relevant to all species including humans.

However, among some participants there was a reluctance to extend this stance to thyroid insufficiencies induced by the hepatic route. In response, some participants pointed to logical inconsistencies if the hepatic mode of action is not deemed relevant when NIS or TPO inhibition are. First, if quantitative differences between the rat and human thyroid systems are the reason for dismissing liver-mediated T4 reductions in the rat as irrelevant, this should equally apply to NIS and TPO inhibition. Second, differences in the PXR/CAR systems between rats and humans cannot be the reason for dismissing observations in the rat, as the human receptors are similar to the rat. There are also no substantial differences between rat and human UDPGT enzymes responsible for conjugation of thyroid hormones.

Concerns were expressed that the existing guidance on interpreting liver-mediated thyroid insufficiency for regulatory decision making is biased in favour of protecting against false positives, when from a public health point of view, protection against false negatives should be imperative. These points could not be resolved, but it was agreed that the technical guidance on interpreting increased T4 clearance *via* hepatic enzyme induction in rodents should be revisited in the context of this discussion. The group agreed that increased T4 clearance in rodents as a result of hepatic Phase II enzyme induction cannot be regarded as irrelevant for humans. It should be presumed to be relevant until additional data with other species indicate the opposite.

Group 2b dealt with the same topic by distinguishing between effects on thyroid and other hormones (*e.g*. estrogens or androgens) resulting from the induction of hepatic enzymes and those resulting from frank liver toxicity. Group 2b agreed that the WHO/IPCS definition of adversity given above does not distinguish between primary and secondary toxicity. Direct effects on the liver will ultimately affect the thyroid system as well as other endocrine axes. A distinction was therefore made between a substance that induces direct liver toxicity and effects on hormone levels secondary to that toxicity and effects on hepatic enzymes. The former would be considered a liver toxicant whereas the latter should be considered an endocrine disruption mechanism. The logic for this view was that the degradation pathways of hormones, and therefore increased hepatic conjugation reactions, are considered key events in maintaining homeostasis, as important as hormone synthesis. The same applies to extra-hepatic thyroid hormone inactivation which can occur *e.g*. in gastrointestinal stromal tumours or as consumptive hypothyroidism through enhanced thyroid hormone degradation *via* type 3 deiodinase.

Consequently, pathways leading to hormone insufficiency have to be deemed both an endocrine-relevant mechanism and relevant to humans, unless information to suggest otherwise is available. It was also noted that this interpretation is relevant to other hormones such as sex hormones and there is no rational basis for a different interpretation pertaining solely to thyroid hormones. During the plenary discussion it was noted that the WHO/IPCS definition of an endocrine disruptor and the draft EU criteria include the necessity for the demonstration of an adverse effect (for example, carcinogenicity, reproductive toxicity).

Thyroid cancer

Group 2a considered trends in thyroid cancer and the role of TSH in these trends. The associations reported in cross-sectional studies were recognised, but it was pointed out that prospective epidemiological studies are needed to further elucidate the role of TSH as a potential causal factor.

The group then discussed the existing USEPA/IARC guidelines which presume that rodent thyroid carcinogens may pose a hazard to humans. The group concluded that, in the absence of data to the contrary, rats should be assumed to be equally sensitive as humans, in line with USEPA/IARC guidance.

Groups 2a and 2b discussed temporal trends in thyroid cancer diagnoses. Thyroid tumours are the most frequent tumour in an endocrine tissue and they are known to be sensitive to both environmental factors and nutritional factors such as iodine or selenium intake. Further, the thyroid is a vulnerable, slowly proliferating tissue. The increase in thyroid papillary tumours was recognised by IARC where a thyroid cancer working group has been set up to investigate the factors underlying the dramatic increase in thyroid carcinomas. It was highlighted that there is a significant contribution of increased awareness and screening to increased trends in diagnosis, as reported in a 2016 IARC publication that was mentioned in group 2a. However, it was noted that there is a lack of clarity in the scientific literature as to whether screening alone can explain the increase in thyroid papillary tumours.

There was general agreement that there is a need to revisit the evidence base on the relevance of the rat model for thyroid cancer (IARC 1998 report), particularly the potential role of the strain of rat used in experiments as large differences can be observed with different strains of mice. The group also noted that it is difficult to distinguish between follicular and papillary tumours in rodents.

Following the Chernobyl incident, an increase in thyroid cancers was observed in children that had been exposed to radiation *in utero* and the prenatal phase is a known sensitive window for thyroid cancer. Group 2b noted however that there is currently no validated carcinogenesis test to detect thyroid cancer in offspring exposed prenatally.

Some discussions took place within the group related to various definitions of carcinogens and whether this would include both tumour initiators and substances that promote proliferation. Group 2b also noted that not all thyroid tumours arise from an ED mechanism.

Group 2b concluded that at present too little is understood of the etiology of thyroid tumours to conclude on the relevance of the rat model for humans. As a result, the rat should be assumed relevant to humans by default – a stance similar to that of group 2a.

Relevance of rat models for human hazard and risk assessment

Further discussions about the human relevance of the rat model to humans took place within group 2b. It was quickly agreed that the rat is more sensitive than the healthy adult human, but not uniquely sensitive. The difference between rat and human is quantitative not qualitative. Similar conclusions were drawn in Group 2a.

Quantitative differences between the rat and the human in terms of thyroid hormone storage were discussed further. The 'buffer' argument was found not to be applicable to sensitive subgroups such as the unborn child, as fetuses do not yet have thyroid hormone storage capacity, or iodine-deficient adults, as their TSH is already elevated. Further, the group noted that in the case of chronic exposure, this buffer may result in increased latency rather than differences in sensitivity. In neonates, the relevant storage pool of thyroid hormones is the thyroid itself rather than peripheral storage in the blood.

8.3.3 Discussion groups 3 a b : Thyroid systems across taxa: the relevance of mammalian data in environmental assessments and of non-mammalian data in human health risk assessment

The suggested discussion topics for groups 3a and 3b were:

1. In which respect can data from rodent studies be used to inform about effects on rodents/mammals in the environment?
2. In which respect can data from amphibian or fish studies be used to inform about possible effects in rodents/mammals?
3. When and how during testing should we take into consideration that the thyroid hormone system is highly conserved between taxa?
4. Should current testing guidelines be enhanced by adding additional endpoints and assays?

Use of laboratory rodent data for purposes of ecotoxicological risk assessment in mammalian species

Participants considered that observations of thyroid disruption in rodent laboratory studies are useful for the identification of thyroid disrupting properties in other wildlife mammalian species. Such findings would raise concerns, especially when considering the well-known similarities in modes of action between taxa. The extrapolation of laboratory findings in rodent to the wildlife population may be more difficult since it requires further consideration on the relevance of such findings at the population level.

Group 3b also considered whether data from rodent studies could be used to inform about possible effects in other taxa, e.g. fish or amphibians. Participants agreed that such data would raise concerns for other taxa, and that mode of action data from mammalian laboratory species might trigger additional testing in other taxa, *e.g*. birds, fish or amphibians.

However, participants cautioned that differences in exposure routes (*e.g*. dermal in amphibians *versus* oral in rodents) and biological differences (presence of the placenta in mammals) might complicate simple species-species extrapolations. There are also significant differences in absorption, distribution, metabolism and excretion.

Use of non-mammalian data for purposes of human hazard and risk assessments

In view of the conservation of the basic features of the thyroid system across taxa, both groups concluded that data from non-mammalian tests can be used to inform on the mode of action of putative thyroid disrupting chemicals. This is strongly supported by the observation that most amphibian thyroid disruptors have elicited positive responses also in rodent tests.

However, both groups considered the issue to be more complicated when it comes to assessing adversity, mainly because of the great care with which species-species extrapolations have to be conducted. Other complicating factors are differences in the exposure routes between *e.g*. amphibians (dermal more relevant) and mammalian species.

Improvements of existing test guidelines from the ecotoxicology perspective

There were extensive discussions of the merits or otherwise of including measurements of thyroid hormone levels in non-mammalian tests. The relevance of such measurements depends on the life stage considered. For example, there will be a high variability during amphibian metamorphosis, with correspondingly low sensitivity. Another question concerns the ways in which such test results should be interpreted. Participants felt that the measurement of thyroid hormone levels is of considerable value in terms of assessments of modes of action, but would not provide additional information in terms of adversity at population level.

Participants of group 3b made detailed recommendations on the inclusion of thyroid hormone measurements in relation to specific assays and tests.

For the AMA, the feasibility was judged as low, and most participants did not rate inclusion as of sufficiently high priority.

For the LAGDA, thyroid hormone measurements were judged to be feasible in plasma, together with determinations of vitellogenin levels, but the majority of participants did not rate the priority as high.

In tests on fish, determinations of thyroid hormones were deemed practical, both in plasma and in fish homogenates. Most of the group maintained that if other thyroid endpoints are included (*e.g*. eye development, swimbladder function, lateral line) it could support the interpretation of data (provide MoA information). One member doubted the current need because of difficulties in interpretation of results (lack of understanding of importance of crosstalk between different hormonal axes for suggested endpoints in fish).

Participants recommended the development of behavioural tests, as changes in behaviour may be downstream effects of thyroid disruption. This was regarded as an important endpoint for adversity of effects which can help to fill the gap between modes of action and adverse effects in terms of population dynamics. However, more research is required before implementation is possible, *e.g.* to identify behavioural effects directly linked to thyroid disruption. Relevant behavioural endpoints to consider include startle response, nesting, feeding behavior and 'swim-up', indicative of swim bladder inflation in fish.

The evaluation of specific thyroid-dependent physiological processes in wildlife species (*e.g*. seasonality, smolting, hibernation) was also seen as desirable, as was the incorporation of swim bladder inflation and eye development as an endpoint in fish early life and generational tests.

Participants pointed out the importance of controlling iodine and selenium supply during testing.

It was also recommended to add thyroid-relevant endpoints to tests on fish since these tests cover a wide range of life stages, with easy wins, especially with the medaka.

The possibility of determining gene expression levels of direct targets of thyroid hormone action, such as Klf9/Bteb, or other thyroid responsive genes regulated by negative feedback, *e.g.* TPO, NIS was also discussed.

Participants discussed the desirability of integrating multiple endpoints for thyroid disruption in non-mammalian tests.

This was approached from several angles, firstly, in terms of multiple endpoints representative of the HPT axis in a single test system. To a degree, this is already realised with the LAGDA. Some participants found it helpful to include liver endpoints in the AMA.

Secondly, the topic was discussed in the sense of the same endpoint in multiple species. The group saw the introduction of thyroid endpoints in fish tests as relevant and worthy of prioritisation. For well established fish test species such as the fathead minnow, thyroid histology could be a valuable endpoint.

Thirdly, participants considered the possibility of investigating multiple endocrine systems in the same species, going beyond the thyroid system. This was seen as a fascinating perspective, but one that requires considerable research and development efforts.

Finally, the topic of maternal transfer of thyroid hormones and of contaminants in non-mammalian test species was discussed. There was a degree of uncertainty as to the importance of maternal transfer in such systems, but a consensus emerged that more research is needed to address this issue.

8.4 Contours of agreements across discussion groups in plenary debates

In plenary sessions, all discussion groups presented summaries of their deliberations for debate and criticism.

Following considerable discussion, all groups came to a consensus regarding the interpretation of changes in serum T4 levels. If such changes occur, for any reason, including altered hepatic clearance, they should be considered a thyroid effect, unless the mechanism of altered clearance (*i.e*. the specific enzymes or pathways impacted) can be determined not to be relevant to humans or wildlife of concern.

All groups also came to the view that the current OECD testing guidelines are insufficiently equipped for capturing all recognised thyroid disrupting effects, and should be updated accordingly. This applies to both, tests intended for human-relevant assessments, and systems for ecotoxicological evaluations. Several discussion groups made specific suggestions for such updates (see above).

9. CONCLUSIONS

This workshop dealt with issues that surround the interpretation of findings from studies relevant to the identification of thyroid disrupting substances. It also identified gaps in the test methods that currently exist for the identification of thyroid disrupting substances.

In the course of the workshop it became clear that the interpretation of test results with thyroid disrupting agents is most often based on an "idealised view" of the thyroid system which is not always in line with experimental observations. In this idealised view, decreases in circulating TH should give rise to increases in TSH which in turn stimulate the thyroid to synthesise TH, thereby restoring the original hormone serum concentrations.

Accordingly, suppressions of TH serum concentrations seen in the wake of exposure to certain substances are expected to also lead to elevated TSH concentrations. When this expectation is not met, the relevance of decreased TH is often called into question, with follow-on debates about the adversity of TH suppressions and the implications for identifying a substance as thyroid disruptor.

However, there are examples in which TH decreases following chemical exposures are not accompanied by correspondingly rising TSH serum concentrations. Sometimes, reduced TH concentrations leave TSH entirely unchanged, in other cases, sustained supression of TH can even lead to decreases of serum TSH.

Furthermore, research in the field of thyroid disruption during the last decade has shown that autonomous regulation of thyroid hormone action at the tissue level, without involvement of the HPT axis, can play an important role, and can affect the organism without the corresponding changes in serum thyroid hormones.

In conclusion, altered TH and TSH levels cannot be seen as the only definitive marker of thyroid hormone action, or of thyroid disruption.

These insights should have significant consequences for the interpretation of data in the context of identifying a substance as thyroid disruptor (and by extension, as an endocrine disruptor). In order to properly identify all thyroid disrupting chemicals, information in chemical dossiers should be supplemented by data on down-stream effects of an adverse nature, such as effects on the developing brain, or on other TH target organs.

However, the current test guidelines in the OECD Conceptual Framework for endocrine disruptors (e.g. TG 421/422, 426, 443) lack such parameters indicative of down-stream adverse effects diagnostic of thyroid disruption. This hampers the reliable identification of substances as thyroid disruptors.

Important perspectives for improving test guidelines are the inclusion of endpoints for the identification of adverse effects on brain function and brain morphology in TG 421/422, 426 and 443, since traditionally used behavioural assays may not be sensitive enough to identify all thyroid disrupting chemicals. Also necessary is the inclusion of endpoints representative of TH action at the cellular level, *e.g*. nerve cell migration and differentiation, or altered expression of TH-dependent genes. Some of these gaps can be filled by including standard endpoints in existing test guidelines (e.g. by making determinations of T4 levels mandatory in TG 407, 408), others require the implementation of new endpoints as well as research and development efforts.

Also notable is a lack of internationally validated *in vitro* assays in the OECD Conceptual Framework. Such data can shed light on important mechanistic aspects of thyroid

disruption (*i.e*. whether a substance is capable of inhibiting NIS, TPO or of displacing TH from serum transporter proteins, etc.).

A strategy for developing improved or novel endpoints important for detecting thyroid disruptors could unfold at the following levels:

- Thyroid histology improvements could be made according to guidelines of the American Medical Association developed for human clinical studies, taking account of improvements in automisation.
- The timing of exposure should be extended, emphasising developmentally exposed animals and including endpoints such as cortical heterotopias and oligodendrocyte differentiation.
- The set of down-stream effects should include brain morphology, especially the developing brain (see above), gene markers of brain development, and TH effects on lipid metabolism and the cardiovascular system.

Until these gaps are filled, it is necessary to evaluate substances on the basis of incomplete data regarding thyroid disruption. In this context, findings of diminished serum TH concentrations in animal studies should be taken as predictive of adverse effects in humans. Such observations should act as a trigger for further testing. However, if further testing cannot be conducted, and more conclusive data are not forthcoming, findings of diminished serum TH levels could form the basis of regulatory decision making, as this is likely to protect against adverse downstream effects (*e.g*. developmental neurotoxicity) in humans.

More specifically, this stance should be extended to substances that provoke suppressions of serum TH concentrations by inducing hepatic enzymes involved in the clearance of TH in rodents. Such findings should be regarded as relevant for regulatory decisions unless, on the basis of further compound specific information, it is possible to exclude the human relevance of such effects. Existing guidance on dealing with such hepatic effects should be reconsidered and updated.

Observations of thyroid disruption in rodent laboratory studies can be useful for the identification of thyroid disrupting properties in other wildlife mammalian species. Considering the preservation of the thyroid system across taxa, such data would also raise concerns for *e.g*. birds, fish or amphibians, although species-species extrapolations will not be straight-forward due to differences in exposure routes (*e.g*. dermal in amphibians *versus* oral in rodents) and other factors (*e.g*. the presence of the placenta in mammals).

Conversely, data from non-mammalian test species can be used to inform on the mode of action of putative thyroid disrupting chemicals in mammals. This is strongly supported by the observation that most amphibian thyroid disruptors have elicited positive responses also in rodent tests.

10. ANNEX 1: LIST OF PARTICIPANTS

Workshop on Thyroid Disruption

29th- 31st March 2017

List of participants

Country	name	institution
France	Stephanie Alexandre	Anses
Denmark	Anna-Maria Andersson	Rigshospitalet
EU	Niklas Andersson	ECHA
Denmark	Marta Axelstad	DTU
EU	Stefanie Barmaz	EFSA
Interest group	Remi Bars	European Centre for Ecotoxicology and Toxicology of Che
UK	Alice Baynes	Brunel University London
Steering group	Claire Beausoleil	ANSES
Norway	Christine Bjorge	Norwegian Environment Agency
Belgium	Marie-Noelle Blaude	WIV ISP
Slovak Republik	Karol Blesak	Ministry of the Economy
Netherlands	Toine FH Bovee	RIKILT (Institute of food safety)
Ireland	Alan Breen	Department of Agriculture, Food and the Marine
OECD	Patience Browne	OECD
France	Nicolas Chevalier	Centre Hospitalier Universitaire de Nice, Nice
Italy	Annamaria Colacci	Arpae Emilia-Romagna
USA	Kevin Crofton	U.S. Environmental Protection Agency
Netherlands	ZhiChao Dang	RIVM
France	Barbara Demeneix	National Museum of Natural History
Switzerland	Anne-Laure Demierre	Bundesamt für Gesundheit BAG
Denmark	Marianna Dybdahl	DTU
Germany	Éva Fetter	UBA
Austria	Alexandra Fischer	Austrian Agency for Health and Food Safety
France	Frederic Flamant	ENSL (Lyon)
Industry	Alexius Freyberger	Bayer Pharma AG
USA	Mary Gilbert	US Environmental Protection Agency
Steering group	Elise Grignard	EC DG JRC
Netherlands	Betty Hakkert	RIVM
Netherlands	Timo Hamers	VU University of Amsterdam
Denmark	Ulla Hass	DTU
Denmark	Henrik Holbech	University of South Denmark
Denmark	Marie-Louise Holmer	Danish Ministry for the Environment
Austria	Bettina Hrdina-Zödl	Austrian Agency for Health and Food Safety
Japan	Taisen Iguchi	Ozaki National Research Institutes, Yokohama City Unive
Lithuania	Agnė Janonytė	Environmental Protection Agency
EU	Aude Kienzler	DG JRC
Germany	Josef Kohrle	Charité Universitatsmedizin Berlin
UK	Andreas Kortenkamp	Brunel University London
Steering group	Peter Korytar	EC DG ENV
UK	Elizabeth Lawton	Brunel University London
France	Joelle Le Moal	Santé Publique France
Sweden	Margaret Lejonklou	KEMI
Norway	Birgitte Lindeman	
Austria	Anne-Marie Losert	Environment Agency Austria
Interest group	Gwynne Lyons	ChemTrust
Italy	Alberto Mantovani	Instituto Superiore di Sanita
Hungary	Zoltan Marcsek	National Institute of Chemical Safety (OKBI)
UK	Olwenn Martin	Brunel University London
OECD 150 Ecotox	Peter Matthiessen	
United Kingdom	Helen McGarry	Health and Safety Executive
Steering group	Cécile Michel	ANSES
Austria	Marc Müller	Austrian Agency for Health and Food Safety
Steering group	Sharon Munn	EC DG JRC
Sweden	Leif Norrgren	Swedish University of Agricultural Science (SLU)
OECD 150 HH	Jenny Odum	
Slovak Republik	Roman Olha	
France	Jean-Nicolas Ormsby	Anses
Finland	Hinni Papponen	
Industry	Dan Pickford	Syngenta
EU	Francesca Pistollato	DG JRC
Finland	Maarit Priha	
Steering group	Maiken Rasmussen	EC DG ENV
Germany	Vera RITZ	BfR
Belgium	Martine Rohl	FPS Public Health
France	Christophe Rousselle	ANSES
Italy	Maristealla Rubbiani	
France	Laurent Sachs	CNRS, National Museum of Natural History, Paris
EU	Gabriele Schöning	ECHA
Germany	Agnes Schulte	BfR
EU	Andrea TERRON	EFSA
Poland	Lidia Tokarz	
EU	Sander van der Linden	DG JRC
Interest group	Majorie van Duursen	Health and Environment Alliance
France	Catherine Viguie	INRA
Norway	Gro Dehli Villanger	
Netherlands	Theo J Visser	Erasmus University Medical Center
Canada	Mike Wade	Environmental & Radiation Health Science Directorate, H
France	Jean-Louis Wemeau	PUPH d'Endocrinologie de Lille, retraite
WCA Consulting	Louise Youngs	WCA Consulting
USA	Tom R Zoeller	University of Massachusetts

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