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Assessment of endocrine disruptive properties of PFOS: EFSA/ECHA guidance case study utilising AOP networks and alternative methods

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Abstract

Endocrine disruptors (EDs) are chemical substances that interfere with the endocrine system, adversely affecting human health and environment. Legislation with aim to eliminate and ban EDs have been introduced in EU, but the identification of EDs remains challenging and crucial step towards regulation and risk management. A guidance for ED assessment has been recently established for pesticides and biocides in the EU, which heavily relies on traditional toxicological testing *in vivo*. Most notably lacking mechanistic methods for some ED modalities and not covering many other modalities that might be affected by EDs. In this project, we focus on the ED assessment according to the valid legislation and explore the possibility to employ alternative methods to bolster the mechanistic understanding of the ED effects and eventually decrease the need for *in vivo* testing. We selected a well-studied industrial chemical perfluorooctanesulfonic acid (PFOS) to be a model compound in a case study for ED assessment where the EU criteria were applied in the frame of human health risk assessment with focus on thyroid disruption and developmental neurotoxicity. A systematic literature review has been conducted for these effects (Scopus, Pubmed, Embase), and relevant studies were selected by title/abstract screening (RAYYAN) and full-text examination. Selected studies were assessed for reliability (SciRAP), and all relevant data were extracted into a database and assessed by Weight of Evidence (WoE) approach. The initial analysis showed potential endocrine adverse effects and endocrine activity, meeting the ED criteria. The use of mechanistic and alternative methods enhanced the outcomes of WoE assessment. Also, the study provides a great hands-on experience with the most up-to-date development in the area of risk assessment and EDs.

Keywords: PFOS, endocrine disrupter, next generation risk assessment, AOP, NAM

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1. Introduction

The endocrine disruptive substances are one of the major challenges in current EU chemical regulation. There is legislation in place for substances with endocrine disrupting properties in the EU regulations for Plant Protection Products (PPP, Regulation, EC No 1107/2009) and Biocidal products (BP, Regulation, EU No 528/2012). Scientific criteria for identifying endocrine disrupting properties within the PPP and BP legislative frameworks were implemented in guidance developed by the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA) and published in 2018 (ECHA/EFSA, 2018). The European Commission stated the aim of developing a horizontal approach to identify endocrine disruptors (EDs) across different EU chemical legislations (EC Communication, 2018). The criteria for identification of EDs are based on the compounds' ability to cause an adverse effect, presence of an endocrine Mode of Action (MoA) and the adverse effect as a consequence of the endocrine MoA (ECHA/EFSA, 2018). The regulatory assessment of EDs thus requires extensive animal testing to identify toxicological effects, as well as a high level of understanding the toxicity mechanism. The PPP and BP regulations further state that the identification of an ED should be carried out by making use of all relevant data, using systematic review methodology and Weight of Evidence (WoE) approaches. The need for systematic and transparent approaches for collecting, evaluating and integrating toxicological data for health risk assessment of environmental factors, including chemicals, has been recognised during the last years (Whaley et al., 2016).

The focus of this project was to investigate the application of the ED criteria and guidance for PPPs and BPs in other regulatory frameworks, such as REACH. Important part was to explore systematic approaches that maximise the use of mechanistic data from non-animal tests and new approach methodologies (NAMs) to facilitate ED assessment. The main focus was on NAMs using modern *in vitro* methods and biomarker assays including omics studies, the links towards the apical effects *in vivo* and their application in hazard and risk assessment. The fellow did get familiar with the legislation related to the assessment of EDs as well as the most current developments in the field via conducting literature review. Based on the collected information a case study was designed where combination of *in vitro*, *in vivo* and mechanistic data (transcriptomics) is being utilised for an ED and risk assessment and which is currently being conducted.

1.1. ED Assessment

Despite differences in the regulatory requirements across legislations (REACH, cosmetics, plant protection products, biocides, etc.), the current ED assessment approach has largely been built around animal studies as adequate models for prediction of potential adverse effects in humans (Knight et al., 2021). However, it is known and accepted that animal studies alone may fail to predict some adverse effects (Takasuna et al., 2017). In addition, for a long time, there has been an ethical concern with the excessive or avoidable use of experimental animals. Directive 2010/63/EU of the EU on the protection of animals used for scientific purposes unambiguously fosters the application of the principle of the 3Rs (i.e. Replacement, Reduction and Refinement of animal testing) when considering the choice of methods to be used. The EU Cosmetics Regulation has gone furthest and banned animal testing of cosmetics altogether both as finished products and certain categories of regulated ingredients. Our work focuses on the assessment outlined in the ECHA/EFSA, (2018) guidance.

1.2. New approach methodologies

Recent regulatory and research activities emphasise the inclusion of modern mechanistic *in vivo* as well as *in vitro* assays in (eco)toxicological risk assessment, including ED assessment of chemicals. The current regulatory approach for identification of EDs focuses on so-called EATS (oestrogen, androgen, thyroid and steroidogenesis) pathways as targets of EDs and defined MoAs while other potentially relevant MoAs should be considered on a case-by-case basis, depending on available evidence (ECHA/EFSA, 2018). Furthermore, even for the EATS modalities, there is only a handful of validated alternative methods for screening and detection to recognise the endocrine activity, since generally the established methods largely rely on mammalian *in vivo* experiments and histopathology. The *in vivo* tests present ethical and economical concerns as well as scientific doubts since these methods are in many cases used mainly for historical reasons while there are more relevant methods available (Knight et al., 2021). There is a large interest from risk assessors, risk managers, researchers and NGOs for comprehensive assessment of applicability and utilisation of new methods, and the EU has an

expressed ambition to reduce the number of animals used for toxicity testing and research purposes (Directive, EC 2010/63/EU). However, the use of non-animal methods for assessment of chemicals in the regulatory setting requires that the mechanistic data generated from such methods can be reliably linked to the adverse health effect that is being predicted. The adverse outcome pathway (AOP) framework provides a means for increased mechanistic understanding and can be used as structured approach for causally linking early events on molecular and cellular levels to adverse health effects relevant for regulatory hazard and risk assessment of chemicals (Ankley et al., 2010).

There is consensus that the most sensitive window of exposure to EDs is during important periods of development, such as foetal development or infancy (Diamanti-Kandarakis et al., 2009). Exposure to EDs during these periods may cause permanent adverse effects later in life. It is also generally recognised that EDs can interfere with endocrine system in various ways. So far, the focus was mainly limited on a number of endocrine modalities, i.e. EATS. However, it has been shown that other aspects of the endocrine system and physiology can be sensitive to EDs as well (Grignard et al., 2020). There is also increasing evidence showing that EDs can work together to produce additive effects ('mixture effect') so that exposure to a combination of EDs may produce an adverse effect at concentrations at which individually no effect has been observed (Thrupp et al., 2018).

However, knowledge gaps still exist. These relate in particular to issues with the classification and assessment of the potential consequences that might results from exposure to EDs. These relate for example to unknown impact of exposure to EDs on disease development, wildlife and ecosystems. Also, there is the ongoing controversy whether and potentially how some basic toxicological principles such as 'safe threshold' are applicable to EDs (Knight et al., 2021). In this context, there is often only limited understanding of the specific contribution of chemical exposure and the way to separate it from other possible causes of the negative impacts being investigated. There is recognised need for better understanding of the mode of action of the endocrine disruption and need for new methods that will better address those needs (Pistollato et al., 2021).

Major efforts are being made and rapid development is seen in research towards new approach methodologies (NAMs) for chemical safety assessment largely driven by interest in regulatory needs (Moné et al., 2020). The ultimate goal of modernised next generation risk assessment (NGRA) is to develop a new approach in which adverse effects are inferred from upfront mechanistic understanding rather than using extensive animal studies (Luijten et al., 2020). Although animal models are currently the standard in predicting adverse human health effects, the correlation between animal models and human health effects is being questioned. Novel methods that would replace the traditional animal testing include batteries of *in silico* (QSARs, PBPK) and *in vitro* assays that would determine the MoA and allow accurate modelling of expected toxicity. However, lack of validated methods (and robust data to base those models on) as well as lack of funding hinder development of such models (Knight et al., 2021). Another driver for this transition is the long-term desire in general population to minimise animal testing. A better mechanistic understanding of toxicological MoA may provide *in vitro* testing methods that more closely represent human biology and accordingly give more accurate predictions (Krewski et al., 2020). To secure a mechanistic basis, the knowledge of toxicological mechanisms needs to be organised in a systematic and transparent manner. Furthermore, such organisation will reveal where appropriate tools and methods are lacking and further investments are needed.

1.3. Adverse Outcome Pathways

The global aim of shifting towards the development of new assessment methods require maximising the use of existing toxicological knowledge. The AOP framework summarise and makes available knowledge about toxicological pathways. Within the efforts of modernising the chemical risk assessment, the AOP framework has prominent place. It can be the major instrument to support the use and interpretation of non-animal and mechanistic data for drawing conclusions about potential health effects of chemicals, as well as for the identification and assessment of EDs. Essentially, AOPs are linear constructs describing biologically plausible chains of events linking a molecular initiating event (MIE), in which the stressor perturbs the biological system, to a series of intermediate key events (KEs) at different levels of biological organisation. The existing link between an upstream KE and a downstream KE in an AOP is called key event relationship (KER). At the other end, the AOP is anchored by an adverse outcome (AO) at the organism or population level (Knapen et al., 2020). The most promising development is formation of quantitative AOPs (qAOP) that provide detailed quantitative understanding of the relations between KEs which would provide ideal tool for connecting mechanistic information with adversity (Spinu et al., 2020).

2. Description of work programme

2.1. Aims

The main goal of the project was to apply the current regulatory rules for ED assessment in a case study with perfluorooctanesulfonic acid (PFOS) and expand the assessment for utilisation of alternative methods beyond classical mammalian models towards hazard and risk assessment in humans. The aim was to explore the possibility of utilisation of the mechanistic data produced by NAMs within the regulatory criteria for ED assessment and the potential of inferring adversity in humans with limited or no animal data.

2.2. Methods

In the presented case study, we focused on PFOS as a model compound. PFOS was selected because it is a well-studied compound for which we could reliably collect sufficient data on both classical studies (e.g. *in vivo* mammalian) as well as studies using NAMs. PFOS is also generally discussed as an ED in the research community but has not been officially assessed and identified as such according to the regulatory criteria. It was therefore of interest to explore to what extent PFOS fulfil the criteria laid out in the regulations while having sufficient data to explore the use of NAMs within the assessment. In our case study we follow the scientific criteria set in the EFSA/ECHA guidance for ED assessment (ECHA/EFSA, 2018) to collect and evaluate available data. We introduced several advancements compared to the guidance by limiting the focus on specific modality only (Thyroid modality only, to make the project manageable in the given time-frame) and including alternative methods in the assessment.

Vast amounts of literature are available on PFOS, covering many aspects of its toxicity in humans and wildlife. There are also several EFSA opinions available on the health risks of PFOS with comprehensive summary of available data and risk assessment for various toxicities with described effects on neurotoxicity, metabolic disruption, immunotoxicity, developmental toxicity (EFSA CONTAM Panel, 2018, 2020). However, the risk assessment predominantly focuses on mammalian toxicity with limited or no mechanistic data for most toxicities and with almost complete lack of any mechanistic insight for, e.g., neurotoxicity. There is an apparent gap in current risk assessment approaches that needs to be addressed. In recent years PFOS has remained of high interest and new studies exploring the mechanisms of its toxicity are being published continuously. Notably, there are several proposed mechanisms for the neurotoxicity and developmental neurotoxicity that would suggest effects in general populations and might be able to provide biologically plausible link between the effects on molecular level and effects observed in epidemiology studies relating to IQ and other neurological impairments.

2.2.1. AOP network

The search for relative information of PFOS, as well as selection and organisation of retrieved data, was supported by use of relevant available AOPs. As a first step, an AOP-wiki screening was conducted to collect the AOPs that provide information on EATS-mediated toxicity pathways. AOPs relevant for EATS were identified in the AOP Wiki by searching for specific toxicological effects and parameters listed as relevant for ED assessment in the ECHA/EFSA guidance (2018). The identified AOPs were manually sorted and combined into an AOP network at common KEs (details on network construction in Appendix A). For this case study, further refinement was made to focus only on AOPs relevant for thyroid hormone (TH) disruption and developmental neurotoxicity (DNT) and a subnetwork was constructed (Appendix A). The information from the TH and DNT AOP network was used as a basis for identifying relevant search terms and constructing a search strategy to identify relevant toxicity data from the scientific literature related to thyroid disruption and effects on neurodevelopment (list of the KEs and related AOPs in Table 1).

2.2.2. Systematic literature search

Search of available peer-reviewed literature was conducted to collect data to support the proposed endocrine disruption property of PFOS disrupting thyroid hormone balance and ultimately causing developmental neurotoxicity. Specific queries were constructed for individual databases based on information collected from the AOP network and initial literature information. The detailed queries for

individual databases are listed in Appendix B. The search was conducted in widely used scientific literature databases Scopus, PubMed and Embase. Two steps were applied for the studies selection from the search: (1) screening by title and abstract, and (2) full-text examination. Title and abstract screening was independently performed by the fellow and one more reviewer using the RAYYAN tool (https://rayyan.qcri.org/). Differences between the reviewers were resolved through discussion. The included and excluded studies were critically identified after defining the problem formulation (scope, scientific needs/objectives and feasibility and the eligibility (inclusion/exclusion) criteria, according the EFSA systematic review methodology (EFSA, 2010). Studies meeting the eligibility criteria were kept for next screening step. Studies clearly not relevant to the problem formulation or meeting the exclusion criteria were excluded. When exclusion could not be made based on the title/abstract, studies were kept for subsequent full-text examination performed by the fellow. A deep examination at full-text level was then performed by the fellow for the screened studies, where those considered that met the eligibility criteria were included into and classified into epidemiological (as supporting information), *in silico*, *in vitro*, *in vivo* mammalians, and *in vivo* non-mammalians.

Table 1: Table of terms extracted from the available AOPs and literature for the systematic search of information on PFOS and TH disruption and DNT (Detailed queries in the Appendix B)

Associated AOP ID	Event ID	Event type	Event name
[42, 54, 128, 134, 159, 175, 176, 188, 271]	277	KE	Decreased thyroid hormone synthesis
[42, 119, 159, 175, 271]	279	MIE	Thyroperoxidase inhibition
[8, 42, 54, 134, 152]	280	KE	Decreased thyroxine (T4) in neuronal tissue
[8, 42, 54, 134, 152, 159, 175, 176, 366, 367]	281	KE	Decreased thyroxine (T4) in serum
[54]	341	AO	Impairment of learning and memory
[54]	381	KE	Reduced levels of BDNF
[54]	385	KE	Decrease of synaptogenesis
[54]	386	KE	Decrease of neuronal network function
[42, 134, 152, 300]	402	AO	Decreased cognitive function
[8, 42, 134, 152, 300]	756	KE	Altered hippocampal gene expression
[8, 42, 134, 152, 300]	757	KE	Altered hippocampal anatomy
[8, 42, 134, 152, 300]	758	KE	Altered hippocampal physiology
[54]	851	KE	Decrease of GABAergic interneurons

2.2.3. Data collection and evaluation

According to the systematic search method, studies were assessed for relevance against inclusion criteria in two steps: (1) screening of titles and abstracts for relevance to the study question, and (2) full-text examination for the eligibility of studies (EFSA, 2010). Therefore, assessment of relevance at this stage was considered as a confirmation and only two categories (relevant and partially relevant) were included since the not relevant studies were excluded at the previous steps after the literature collection. The relevant studies were then assessed for reliability (inherent quality of the test method and level of reporting) by the online web-tool Science in Risk Assessment and Policy – SciRAP (https://www.scirap.org). SciRAP provides pre-defined criteria and a colour-coding tool aimed to promote structure and transparency in the evaluation toxicity (*in vitro* and *in vivo*) studies for hazard and risk assessment of chemicals. When a study contained both *in vitro* and *in vivo* individual SciRAP evaluations were performed for the endpoints. The SciRAP score was converted into Klimisch reliability criteria (reliable without restriction, reliable with restriction, not reliable and not assignable) which were then use for the purpose of the WoE assessment according to systematic approach previously described in Ingre-Khans et al. (2020).

2.2.4. Weight of evidence assessment

The extracted parameters along with the study quality assessment scores were assembled into lines of evidence for the groups (a) thyroid-related endocrine activity, (b) thyroid and nervous system adversity, and (c) general toxicity. Each group was subdivided into categories based on the nature of the data addressing specific endpoints or MoAs. Each individual line of evidence was assessed

considering the quantity and quality of both the studies and the included parameters, as well as their coherence dose/concentration–response, consistency among studies and repeatability for the line of evidence. Each line of evidence was assessed, and evidence was categorised into five groups: Strong, Moderate, Weak and No evidence for an effect and No evidence available. The evidence assembled was then used to draw conclusion whether sufficient evidence is available for endocrine disruptive effects for the proposed modality of thyroid disruptions and adversity of developmental neurotoxicity.

2.3. Activities

The fellow participated in regular group meeting during the placement at IMM, KI and engaged in discussion with multiple out of the team colleagues. Despite the challenging circumstances and limited time when personal meetings were possible, fellow also participated in following activities:

- Preparation and moderation of a IMM organised webinar Next Generation Approaches for Regulatory Assessment of Endocrine Disruptors, October 28, 2021
- Meetings and discussions with collaborators from University of Antwerp discussing the AOP networks and further applications, October 2021–January 2022
- Participation in webinars focusing on NAMs
    - Endocrine disruption as a mechanism of developmental Neurotoxicity (DNT), September 15, 2021, hosted by International Neurotoxicology Association, (virtual; https://www.neurotoxicology.org/ina-webinars/)
    - 10th Annual Meeting of the ASCCT, 'Practical applications of new tools in toxicology' October 12-14, 2021 (virtual; https://www.ascctox.org/annualmeeting)
- Planning with colleagues from unit about next generation risk assessment report that should be prepared in future.
- European commission Third Annual Forum on Endocrine Disruptors (https://ec.europa.eu/environment/events/third-annual-forum-endocrine-disruptors_en).
- Activities related to oversight and management of student projects of Sara Caccia (master project) and Linus Wiklund (PhD project) within the unit.
- Attending lectures on relevant topics within IMM.

3. Conclusions

Exploring and implementing innovative non-animal-based approaches requires a long-term and focused development effort that is complemented with well-planned and funded research. How to reach implementable outcome is not yet clear. To apply these approaches once developed, the relevant EU legislation and guidance as well as regulatory practice will have to be updated. In particular, combining NAMs with standard methods to strengthen the evidence for regulatory needs, i.e. read-across and WoE, and their potential use as screening and priority setting tools to identify compounds of regulatory interest. There is also the opportunity re-consider the safety evaluation in general to adopt predictive toxicology especially the costs, the ethics and the usefulness of animal studies, and to expand the role of monitoring exposure post market with the aim to achieve at least the same level of protection without cross validation to animal studies.

It should be possible to improve the identification of adverse effects that are not addressed by current validated toxicology studies and at the same time aim towards reducing the use of animals for toxicity testing. In this regard, the AOP concept is an important development. It should help to formalise toxicological base and evidence for development of a testing battery based on in silico and *in vitro* methods to allow predictive toxicology and inform on the adversity or at least help with prioritisation. The overall conclusion from predictive toxicology for a compound must be clear on how it was made and what is the associated level of uncertainty. A key challenge for new safety assessment approaches is therefore to agree on what constitutes adequate evidence to justify non-animal-based safety testing and assessment approaches. One of the main concepts is the acceptance of non-animal data as reliable predictors for health effects in humans. The key issues to promote that are to develop and employ standards for NAM data, including the biological relevance of the methods, and data integration approaches to conclude on the safety assessment. Additional challenge is adapting the training and skills of future risk assessors that will need better understanding of the new methods and concepts. That brings substantial requirements on the risk assessors in terms of expertise in very wide field from QSARs and *in vitro* assays to biomarkers and omics. It will require

further resources and coordinated approach to prepare the next generation risk assessors that will be a match to the next generation risk assessment.

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Abbreviations

AOP	adverse outcome pathway
DNT	developmental neurotoxicity
EATS	oestrogen, androgen, thyroid hormone and steroidogenesis
ED	endocrine disruptor
KE	key event
KER	key event relationship
MIE	molecular initiating event
MoA	mode of action
NAM	new approach method
NGRA	next generation risk assessment
PFOS	perfluorooctanesulfonic acid
qAOP	quantitative adverse outcome pathway
TH	thyroid
WoE	weight of evidence

Appendix A – AOP Network construction

AOPs from the OECD AOP-Wiki 2.4 were investigated manually to develop the derived AOP network. The full list of linear AOPs available in the AOP-Wiki database was the starting point for our search. The linear AOPs were collected April 9th, 2021, and the last check was performed in July 2021. The list of linear AOPs relevant to EATS modalities was manually extracted by assessment of the Abstract, the Background (if present) and the Overall Assessment sections for each extracted AOP as well as analysing their graphical representation displayed into the AOP-Wiki. An additional refining step was applied to isolate from the EATS-related AOPs list the ones that could be considered specifically Thyroid-related. At this purpose, another single-concept querying of the AOP-Wiki database was performed: the sorting procedure started from the previously collected AOPs retrieved typing the general key word 'Thyroid' into the AOP-Wiki database. A new systematic search in the AOP-Wiki followed employing only those parameters that in the ED GD are reported as indicative of thyroid modalities (Indicative of T modality) as search terms for developing a TH-related AOPs list. The list of linear AOPs manually extracted from the AOP-Wiki database applying the strategy was further inspected to exclude those AOPs that, even if retrieved through a single-concept querying based on TH-parameters, were erroneously included in the selection. This procedure was again performed by assessing the Abstract, the Background (if present) and the Overall Assessment sections for each extracted AOP as well as analysing their graphical representation displayed into the AOP-Wiki. Eventually, matches among the collected AOPs and the previously refined EATS-related AOPs list were highlighted obtaining the final refined TH-related AOPs list. The search was performed in the AOP-Wiki database on 26 May 2021, and the last check was performed in July 2021.

Cytoscape 3.8.2 (https://cytoscape.org/) was employed to model both the EATS-related and TH-related AOP-networks. This open-source software platform enables its users to generate a wide variety of networks either manually or importing data tables (e.g. Excel spreadsheets containing interaction-data between biological pathways); additionally, the program provides a basic set of features for data integration, analysis, and visualisation. The full content of the AOP-Wiki is available in an XML format (https://aopwiki.org/downloads/aop-wiki-xml.gz). Additional files with specific subsets of content are also accessible for users who don't wish to analyse the full XML documents; however, these files are daily updated and replaced with no permanent backups. In this project, for the generation of the EATS-related and TH-related AOPNs the download and use of the XML files was used in combination with information from tab-separated files (.tsv) that were downloaded from the AOP-Wiki platform (https://aopwiki.org/info_pages/5) on 4 July 2021. Since these files have no permanent backups in the AOP-Wiki platform, their original version will be conserved and made available for reproducibility purposes. For extraction of data from the xml file (downloaded on 4th July, 2021) modified R code from Pollesch et al. (2019) was used to extract information about biological level relevance and AOP status information.

The downloaded documents were processed employing KNIME Analytics Platform (https://www.knime.com/knime-analytics-platform), an open-source software that offers visual workflows for data analytics with an intuitive, drag and drop style graphical interface and for which no complex coding is required. Among all, KNIME enables to combine and handle data in simple text formats. Using KNIME's features, in both EATS- and TH-related tables a new attribute named 'Associated AOP Ids' was created and assigned to each listed KE and added to the downloaded tables in a dedicated column. This newly generated information lists in square brackets all the concatenated AOP Ids the referred KE belongs to and is essential to obtain an automatic AOPN mapping employing Cytoscape. Eventually, data coming from the three downloaded.tsv Files were merged in two distinct Excel tables (one for EATS-related and one for TH-related AOPs). Those tables were based on the Key Event Relationships File to which data from Key Events and Key Event Components File were attached (link-up was set for the Upstream Event Id in each KER and Event Id in the Key Events file; KEs without KERs were attached at the end of the table). Two additional data columns were added to the tables named 'Label' (combining the following attributes taken from Key Event file: Event Id, Event Type, Event Name and Associated AOP IDs) and 'Rel. Label' (combining the following attributes taken from KE Relationships File: Relationship Id, Associated AOP Ids). Importing in Cytoscape the unified Excel spreadsheets, two networks were automatically generated and graphically displayed using the program's default mapping features. These were promptly customised applying a series of graphical changes working manually on Cytoscape's Style interface. An accurate refinement of both networks was obtained through the following passages: first, a specific geometrical shape was addressed to each node according to its KE Type property as shown in Figure. An edge target arrow shape was then defined to enable the visual

understanding of the AOP of interest and facilitate the KEs along the path itself. The edges' look was further made to correspond to the KER's Adjacency applying a continuous and a dashed line fashion for adjacent and non-adjacent relationships respectively (Figure A.1).

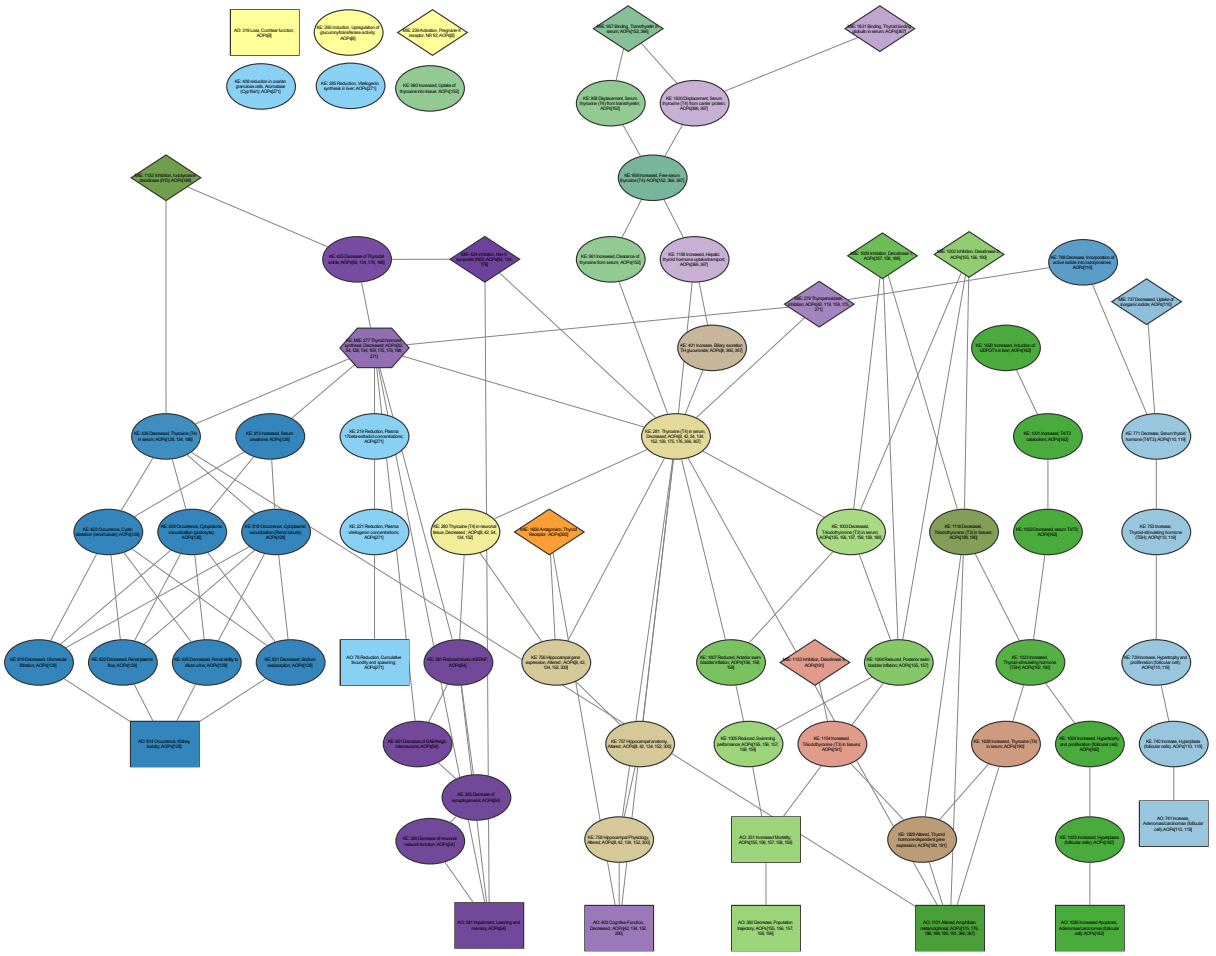

Figure A.1: Putative AOP Network of all KEs associated with AOPs that are related to Thyroid hormone disruption and Developmental neurotoxicity. The network was generated by using data from AOP-wiki (Accessed: 4 July 2021)

Appendix B – Systematic literature search

Table B.1: Basis of the search query derived from the selected AOPs

Database	Scopus	PubMed (Abstract Sifter)	Embase
CAS	CASREGNUMBER(1763-23-1)	–	–
Compound	ALL(PFOS OR "Perfluorooctanesulfonic acid" OR "Perfluorooctane sulfonic acid" OR "heptadecafluorooctane sulfonic acid" OR "Perfluorooctane sulfonate"))	("1763-23-1" OR PFOS OR "Perfluorooctanesulfonic acid" OR "Perfluorooctane sulfonic acid" OR "heptadecafluorooctane sulfonic acid" OR "Perfluorooctane sulfonate")	('1763-23-1':ti,ab,kw OR pfos:ti,ab,kw OR 'perfluorooctanesulfonic acid':ti,ab,kw OR 'perfluorooctane sulfonic acid':ti,ab,kw OR 'heptadecafluorooctane sulfonic acid':ti,ab,kw OR 'perfluorooctane sulfonate':ti,ab,kw)
TH terms	(TITLE-ABS(thyroid OR "thyroid hormone*" OR "thyroid gland" OR "thyroid peroxidase" OR "thyroperoxidase" OR "iodide peroxidase" OR "thyroxine")	((thyroid OR "thyroid hormone*" OR "thyroid gland" OR "thyroid peroxidase" OR "thyroperoxidase" OR iodide peroxidase OR "thyroxine")	(thyroid:ti,ab,kw OR 'thyroid hormone*':ti,ab,kw OR 'thyroid gland':ti,ab,kw OR 'thyroid peroxidase':ti,ab,kw OR 'thyroperoxidase':ti,ab,kw OR 'iodide peroxidase':ti,ab,kw OR 'thyroxine':ti,ab,kw
DNT terms	TITLE-ABS(hippocampus OR hippocampal OR synaptogenesis OR "neuronal network*" OR "cognitive" OR "GABAergic interneuron" OR "neuronal tissue*" OR learning OR memory OR bdnf OR "brain derived neurotrophic factor*"))	(hippocampus OR hippocampal OR synaptogenesis OR "neuronal network*" OR "cognitive" OR "GABAergic interneuron" OR "neuronal tissue*" OR learning OR memory OR bdnf OR "brain derived neurotrophic factor*"))	hippocampus:ti,ab,kw OR hippocampal:ti,ab,kw OR synaptogenesis:ti,ab,kw OR 'neuronal network*':ti,ab,kw OR 'cognitive':ti,ab,kw OR 'gabaergic interneuron':ti,ab,kw OR 'neuronal tissue*':ti,ab,kw OR learning:ti,ab,kw OR memory:ti,ab,kw OR bdnf:ti,ab,kw OR 'brain derived neurotrophic factor*':ti,ab,kw) NOT [medline]/lim