Toward an AOP network-based tiered testing strategy for the assessment of thyroid hormone disruption

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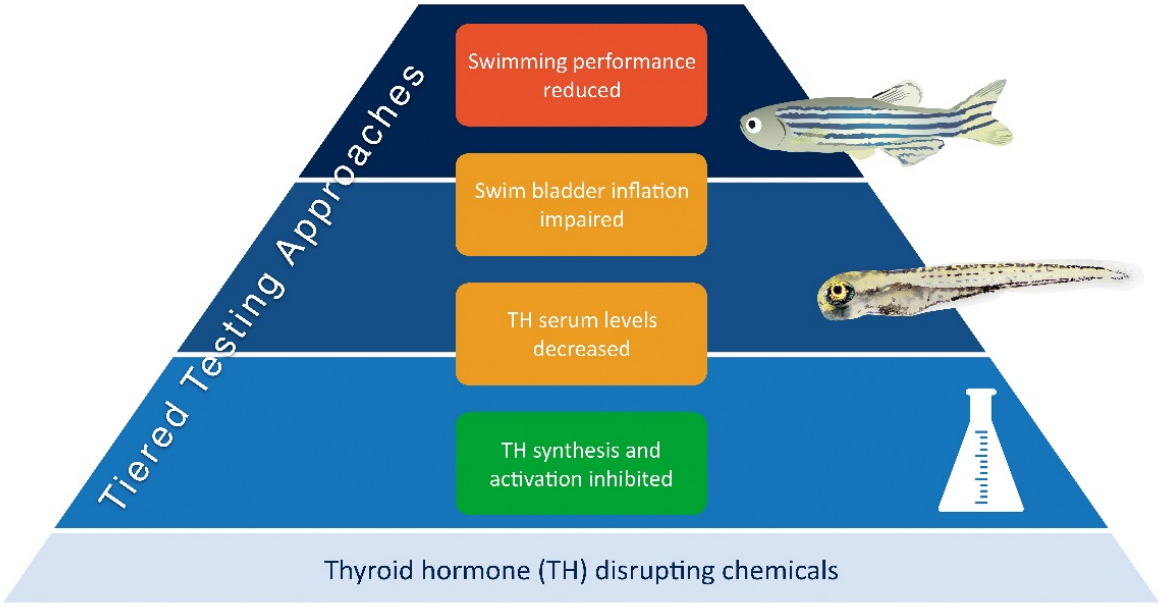
Abstract

A growing number of environmental pollutants are known to adversely affect the thyroid hormone system, and major gaps have been identified in the tools available for the identification, and the hazard and risk assessment of these thyroid hormone disrupting chemicals. We provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. We demonstrate how a suite of assays covering biological processes involved in the underlying toxicological pathways can be implemented in a tiered screening and testing approach for thyroid hormone disruption, using the levels of assessment of the OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals as a guide.

Keywords: thyroid hormone disruption, tiered testing, adverse outcome pathway, fish, early-life stages

Graphical abstract



1. Background

Screening and testing programs for the assessment of endocrine-active chemicals are being implemented throughout the world [1]. Endocrine disruption by chemicals is not restricted to the sex hormone and reproductive systems, but also includes thyroid hormone disruption. Thyroid hormones (TH) play a crucial role in the regulation of vertebrate development and homeostatic processes related to growth and energy metabolism, and a growing number of high-profile environmental pollutants has been shown to adversely affect the hypothalamic-pituitary-thyroid (HPT) axis [2, 3]. While there are many models and assays available for detecting chemicals that impact the hypothalamic-pituitary-gonadal axis, such as estrogen and androgen receptor agonists and antagonists, major gaps have been identified in the tools available for the hazard and risk assessment of HPT-active substances [4, 5]. The scientific community is therefore challenged with developing new or improved testing approaches to evaluate TH disruption. A substance is considered as having endocrine-disrupting properties if (1) it shows an adverse effect, (2) it has an endocrine mode of action, and (3) the adverse effect is a consequence of the endocrine mode of action [6-8]. A high level of uncertainty relative to the causal relationship between mechanistic responses and apical, adverse outcomes is one of the main limitations of the current test systems for the identification of endocrine-disrupting chemicals and for evaluating endocrine hazard and risk [9]. This is of particular importance in the case of TH disruption since the adverse effects associated with disruption of the HPT-axis are often associated with general biological processes (e.g., embryonic development, energy metabolism) that can be affected by many different toxicological pathways, including mechanisms unrelated to the thyroid system. A second limitation of current test methods is that they are costly, time-consuming, and animal intensive [10]. To address these various challenges, tiered testing approaches for the assessment of endocrine-active chemicals have been developed by different countries and international organisations, in which lower tier data (e.g., *in silico*, *in vitro* or *in vivo* data) are used to decide whether more elaborate, resource-intensive higher tier *in vivo* tests are needed to demonstrate adverse apical effects. The U.S. Environmental Protection Agency's (USEPA) Endocrine Disruptor Screening Program (EDSP) and the

Organisation for Economic Cooperation and Development (OECD) Conceptual Framework (CF) for the Testing and Assessment of Endocrine Disrupting Chemicals [7, 11] are among the most important examples of well-established tiered testing approaches [9, 12].

The adverse outcome pathway (AOP) framework [13, 14] is, by design, well suited to directly support the development of tiered testing approaches by providing evidence for the association between a toxicological pathway perturbation and downstream responses [9]. An AOP summarizes available empirical evidence demonstrating the mechanistic, causal linkages leading from a molecular initiating event (e.g., inhibition of an enzyme involved in TH synthesis) to an adverse apical outcome (e.g., reduced growth). Typically, an AOP is graphically depicted as a diagram which represents the assembly of scientific evidence and mechanistic support underlying the toxicological pathway under consideration. By integrating data across different levels of biological organization, the AOP framework can thus provide support for the mechanistic link between an endocrine-active mechanism detected using *in vitro* or lower tier *in vivo* assays, and potential apical effects measured in higher tier *in vivo* tests [1, 9]. The present paper provides an example of how the AOP framework and associated data generation can address current TH disruption testing challenges in the context of fish early-life stage assays, and fish assays in general. Although standardized and validated fish assays are routinely used in environmental hazard and risk assessment, the current fish test guidelines lack endpoints that are informative of TH disruption [7]. Here, we build upon a recently developed AOP network linking disruption of the HPT-axis in fish to impaired inflation of the swim bladder, leading to reduced swimming performance and ultimately survival [15, 16]. We demonstrate how assays covering essential biological processes represented by the different key events of the AOP network can be implemented in a tiered screening and testing approach for TH disruption in fish (see the caption of Figure 2 for more information on the essentiality of key events). The levels of assessment as established by the OECD CF are used as the primary guide for structuring our discussion.

2. Brief description of the AOP network

An AOP network is defined as an assembly of 2 or more AOPs that share one or more key events [15]. The AOP network used in this case example links TH disruption to impaired swim bladder inflation in fish and is mainly based on experimental evidence from studies on zebrafish and fathead minnow including chemical exposures, knockdowns, knockouts and rescue experiments [15-32]. The swim bladder is an internal gas-filled organ found in many bony fish species and typically consists of two gas-filled chambers. The posterior chamber inflates during early development and contributes to the ability of fish to control their buoyancy, while the anterior chamber inflates during late development and has an additional role as a resonating chamber to produce or receive sound [33]. A large body of evidence is available demonstrating the role of THs in swim bladder development and inflation. The AOP network describes how decreased synthesis and/or decreased biological activation of THs leads to incomplete or improper inflation of the swim bladder, leading to reduced swimming performance and ultimately to reduced survival.

Specifically, the AOP network includes two distinct molecular initiating events, corresponding to the inhibition of enzymes involved in the TH metabolism (Figure 1). Thyroperoxidase (Tpo) is the main enzyme involved in TH synthesis in the thyroid gland, and deiodinase (Dio) 1 and 2 are mainly involved in the activation of thyroxin (T4) to triiodothyronine (T3), the most biologically active form of TH. Inhibition of Dio directly results in reduced serum T3 levels, while inhibition of Tpo leads to decreased T4 levels and thus to lower availability of T4 for activation to T3, also resulting in decreased serum T3 levels. As such, reduced T3 levels are a point in the AOP network where different TH disrupting mechanisms converge [15] and which is essential for the progression to different adverse outcomes, depending on life-stage.

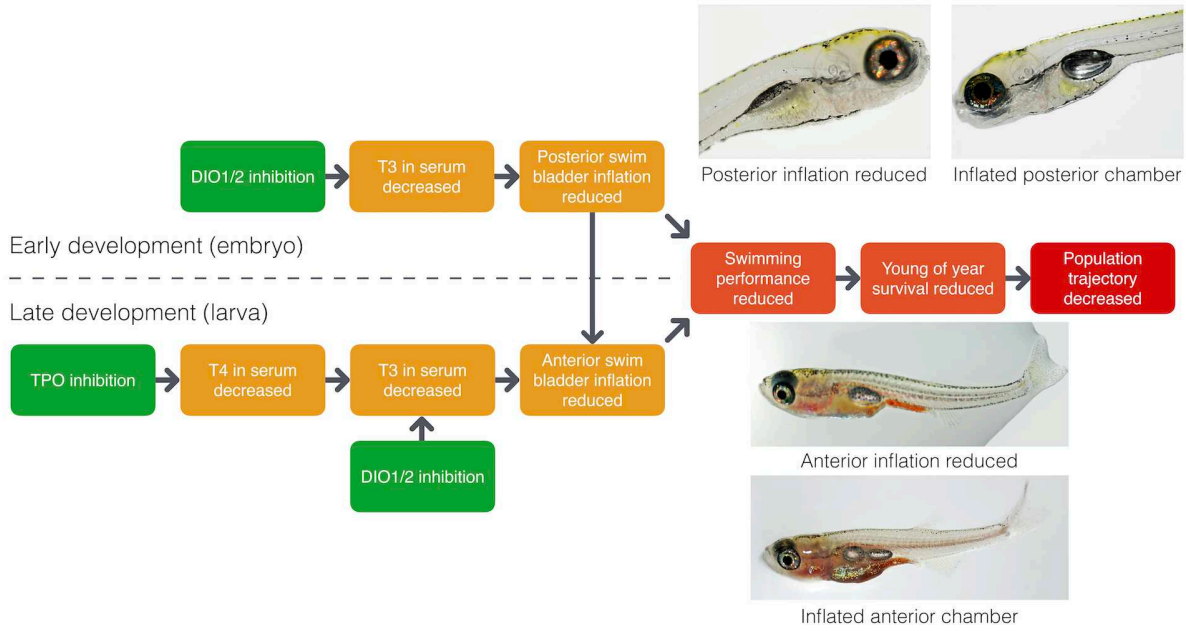


Figure 1. Graphical overview of an adverse outcome pathway (AOPs) network linking thyroperoxidase (TPO) inhibition and inhibition of deiodinase (DIO) 1/2 to reduced swim bladder inflation in fish and subsequent impacts on young of year survival. The AOPs relevant during different life stages are depicted above and below the dashed line [32].

Indeed, specific parts of the AOP network are relevant to different life stages (see Figure 1). The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active [34]. As a result, early developmental processes that are dependent on THs, such as posterior swim bladder chamber inflation, appear to be less sensitive to inhibition of TH synthesis. On the other hand, when maternally derived THs are depleted during late development (larval stage), endogenous TH synthesis becomes more important and inhibition of Tpo interferes with proper inflation of the anterior swim bladder chamber [18, 19, 21, 28]. In all life stages however, the conversion of T4 into T3 is essential. Inhibition of Dio therefore impacts swim bladder inflation in both early and late developmental life stages [21-23, 28, 29]. The anterior chamber develops by budding out of the posterior chamber and thus failure to properly inflate the posterior chamber during early development directly impacts anterior chamber inflation during late development. Impaired swim bladder inflation results in reduced swimming performance [17, 18, 21, 22], an adverse outcome that

134 can affect feeding behavior and predator avoidance, ultimately leading to lower survival probability
135 and population trajectory decline [24].
136
137 3. Toward an AOP network-based tiered testing strategy
138 The OECD is an international organization promoting global cooperation to face modern day
139 challenges in various areas including human and environmental health. In 2002 (updated in 2012 and
140 2018), the OECD released the Conceptual Framework (CF) for Testing and Assessment of Endocrine
141 Disrupters that organizes current methods for screening and testing of endocrine-active substances
142 into 5 levels [11]. Level 1 of the CF relies on existing data and quantitative structure–activity relationship
143 (QSAR) or non-test information to predict the endocrine-active potential of chemicals (Figure 2). Level
144 2 (*in vitro*) and Level 3 (*in vivo*) assays inform whether a substance can interact with endocrine
145 pathways. These assays can be used to screen for possible endocrine activity but are typically limited
146 in their ability to capture all existing endocrine mechanisms and in the observation of adverse apical
147 effects. Level 4 is comprised of *in vivo* assays that provide data on endocrine-relevant adverse apical
148 effects and are typically responsive to more than one endocrine mode of action. Finally, Level 5 assays
149 include full life-cycle tests and multigenerational studies providing more comprehensive data on
150 adverse effects over more extensive parts of the life cycle. Level 4 and Level 5 assays are focused on
151 observing adverse effects and can be used for evaluating both the actual endocrine disrupting
152 properties of substances and their potential risk. It should be noted that within the context of the CF,
153 entering and exiting at all levels is possible and depends on the nature of existing information and
154 needs for testing and assessment [7]. The USEPA EDSP uses a two-tiered approach in which Tier 1
155 screening data, corresponding to CF Levels 1-3, are used to identify substances that have the potential
156 to interact with endocrine systems and Tier 2 identifies and characterizes any adverse endocrine-
157 related apical effects, corresponding to CF Levels 4-5.
158 In 2018, the OECD published an updated version of Guidance Document 150, *Standardised Test*
159 *Guidelines for Evaluating Chemicals for Endocrine Disruption* [7]. This document, originally published in

2012, is intended to provide guidance for evaluating chemicals using standardised test guidelines within the context of the OECD CF. The guidance document provides advice on how to use and interpret the outcome of individual tests/assays and attempts to address the need for a causal linkage between likely mechanisms of endocrine action and resulting apical effects. It also provides a list of assays, including those for the assessment of TH disruption, that could be valuable additions to existing test guidelines but for which currently no formal test guidelines are available. The AOP network described here provides a mechanistic basis for adding a suite of TH disruption-specific assays and relevant additional endpoints (see list of AOP-based assays in Figure 2) for a number of existing fish test guidelines.

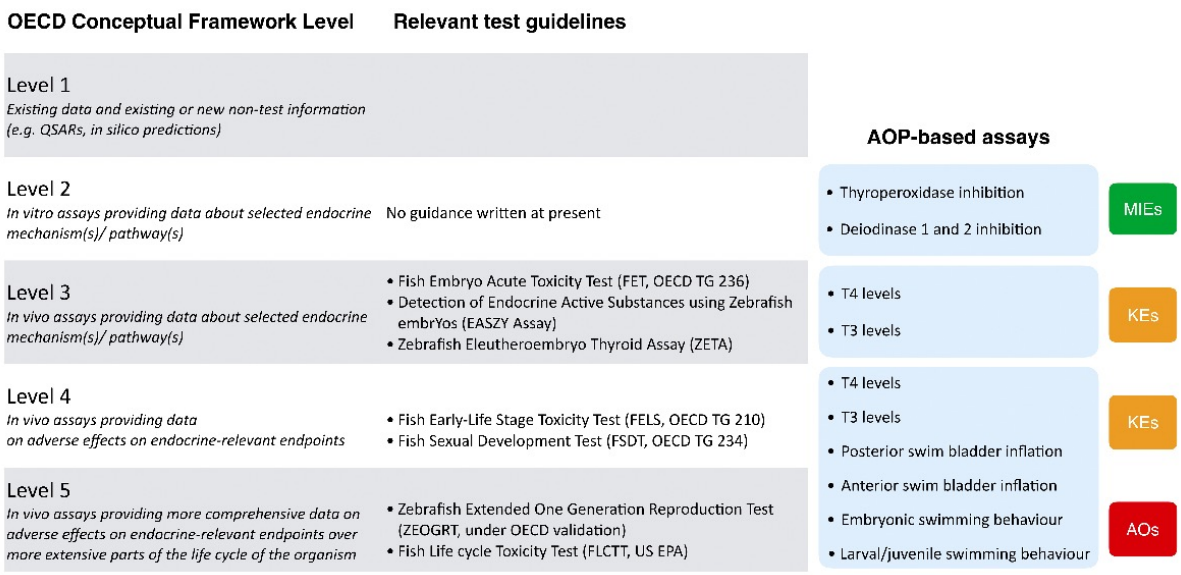


Figure 2. Overview of assays aligned with the thyroid hormone disruption AOP network and how they could be used in a tiered testing strategy based on the Organisation for Economic Cooperation and Development (OECD) Conceptual Framework [7, 11]. Only test guidelines that are directly relevant to zebrafish and/or fathead minnow early-life stages, on which the current AOP network is based, are mentioned. Level 1 is mentioned for completeness. MIE, KE and AO are AOP-specific terms [35]. MIE: molecular initiating event, the point where a chemical directly interacts with a biomolecule to create a perturbation, depicted in green. KE: key event, a measurable change in biological state that is essential, but not necessarily sufficient for the progression from a defined biological perturbation toward a specific adverse outcome, depicted in orange. AO: adverse outcome,

measured at a level of organization that corresponds with an established protection goal and/or is functionally equivalent to an apical endpoint measured as part of an accepted guideline test, depicted in red.

3.1. *In vitro* assays for thyroid activity screening

There are currently no internationally validated test guidelines for *in vitro* assays to screen for thyroid-active substances at Level 2 of the CF (Figure 2). Several international efforts have, however, assessed the availability and readiness of *in vitro* screening assays for thyroid-active chemicals [3, 36]. Important progress has recently been made on the development of assays to evaluate reduced TH synthesis via inhibition of Tpo activity [37] and iodide uptake [38], reduced TH (in)activation by inhibition of deiodinase activity [39], and inhibition of cellular TH uptake [40]. Several of these assays have been applied to large chemical libraries and are currently being added to the USEPA Toxicity Forecaster (ToxCast™) program, a chemical prioritization effort that uses a suite of high-throughput screening assays to rank and prioritize chemicals for future testing, thereby aiding in efficient management and regulation of environmental contaminants. Recently, the extent to which available high throughput screening assays cover the known molecular targets for TH disruption across vertebrates was evaluated. These molecular interactions were then linked to downstream events and adverse outcomes based on a cross-species TH disruption AOP network [41]. In July 2017, the Joint Research Centre EU Reference Laboratory for alternatives to animal testing (JRC EURL ECVAM) launched a validation study to assess a battery of 17 *in vitro* screening methods covering a series of TH disrupting modes of action. Consequently, the scope of assays available for Level 2 of the CF is expected to continue to grow in the near future.

The AOP network links molecular interactions measured in *in vitro* Tpo and Dio inhibition assays with altered TH levels and downstream adverse *in vivo* effects in fish. Here, we use it in a tiered testing strategy to guide the selection of suitable assays and endpoints for the evaluation of adverse *in vivo* effects. Specifically, a positive result in the *in vitro* Tpo and/or the Dio inhibition assay could trigger

fish *in vivo* testing to confirm the occurrence of downstream events along the AOP network including altered TH levels, impaired swim bladder inflation, and altered swimming performance (see sections 3.2 and 3.3). An initial validation case study showed that adverse swim bladder effects could be predicted along the AOP network based on *in vitro* Dio inhibition data [22]. The further development of such predictive approaches, including expanding the quantitative understanding of the relationships in the AOPs where possible [42], would significantly reduce the need for *in vivo* testing in the future.

3.2. *In vivo* assays for thyroid activity screening

Currently, the only non-mammalian *in vivo* assays assessing thyroid-specific endpoints at Level 3 of the CF use amphibians. The Amphibian Metamorphosis Assay (AMA, OECD TG 231, US EPA OPPTS 890.1100) is the most widely-used assay for detecting HPT-active substances, but was recently complemented with the *Xenopus* Eleutheroembryonic Thyroid Assay (XETA, TG 248). None of the current CF Level 3 fish assays include thyroid-specific endpoints.

Fish and amphibian embryo assays have added value for screening purposes compared to *in vitro* assays due to the increased biological relevance gained from using a model organism with an intact HPT axis and ongoing, complex development. In this context, the Fish Embryo Acute Toxicity (FET) test (OECD TG 236) with the addition of TH measurements as thyroid-specific endpoints could be a valuable Level 3 screening assay for TH activity. It should be noted however that since collecting blood from early life stages of fish is often not feasible, whole body TH measurements are typically used as a proxy for serum TH levels [21]. Similarly, TH measurements could be carried out as part of the "EASZY Assay" (*Detection of Endocrine Active Substance, acting through estrogen receptors, using transgenic Zebrafish embrYos*), for which an OECD test guideline was recently drafted. The importance and relevance of determining altered TH levels as an indicator of endocrine activity in *in vivo* assays for TH disrupter screening and testing has already been acknowledged [4, 7]. The AOP network further highlights the critical nature of altered TH levels as a point of convergence for several TH disruption

mechanisms and essential step in the progression towards an adverse outcome. The ZETA (Zebrafish Eleutheroembryo Thyroid Assay) quantifies intrafollicular T4 content as an indirect measurement of TH synthesis in 5 day old zebrafish embryos. It is a first example of a thyroid-specific fish test that has been proposed and is currently being explored as part of the JRC EURL ECVAM validation effort [36, 43]. Viable methods for directly measuring altered whole body TH levels (T4, T3) in fish embryos have recently been developed [18, 19, 21, 23]. Today, the addition of TH measurements to the FET test, and possibly the ZETA and EASZY Assay, for detecting TH disruption screening has therefore become both sensible and achievable. An accurate assessment of posterior chamber inflation and swimming performance however cannot be reliably carried out within the context of the FET test, which has a duration of 96 hours, since the posterior chamber of zebrafish inflates around 120 hours post-fertilisation. Also, many endocrine (e.g., delayed development) and non-endocrine or indirect endocrine (e.g., altered feeding behavior, increased energy expenditure) mechanisms negatively impact growth rate, thereby potentially further delaying posterior chamber inflation. Therefore, we only suggest the addition of TH measurements, and not assessment of swim bladder inflation, to existing fish embryo tests for TH activity screening.

Importantly, assays using fish or amphibian embryos are considered non-animal methods in many parts of the world. For example, non-mammalian vertebrate embryos are not protected until the stage of free-feeding under the current EU legislation on the use of laboratory animals [44]. In a tiered testing approach, *in vitro* and non-animal assays (e.g., fish and amphibian embryo assays) could reduce the need for *in vivo* testing. Naturally, the limitations that have been considered in the debate on the regulatory acceptance of the FET test within the context of the REACH legislation should be taken into account. These include the presence of a chorion during the first few days of the test which may function as a barrier to some chemicals, and the limited xenobiotic metabolism capacity compared to later life stages [45].

3.3. *In vivo* assays for thyroid hormone disruption testing

There are two non-mammalian assays, one with an amphibian and one with an avian species, at Levels 4 and 5 of the CF and Tier 2 of the EDSP that have thyroid-specific endpoints (OECD TG 241, US EPA OCSPP 890.2100). Several fish assays with zebrafish and/or fathead minnow early-life stages are also listed as Level 4 and 5 tests in the CF (Figure 2): the Fish Early Life Stage Toxicity (FELS) Test (OECD TG 210, Level 4), Fish Sexual Development Test (FSDT, OECD TG 234, Level 4), Zebrafish Extended One-Generation Reproduction Test (ZEOGRT, draft OECD TG, Level 5), and Fish Life Cycle Toxicity Test (FLCTT, US EPA OPPTS 850.1500, Level 5). These assays all assess endpoints that are potentially sensitive to, but not necessarily diagnostic of TH disruption (i.e., general adverse effects such as reduced growth that might respond to TH disruption but can also be affected by other toxicological pathways). It has been suggested that new, specific endpoints could be added to these existing test guidelines to increase their diagnostic value for the assessment of TH disruption [4]. Recently, addition of thyroid-related endpoints in OECD fish test guidelines such as the FET and FSDT was included in the OECD's Work Plan for the Test Guidelines Programme (project 2.64).

Neither swim bladder inflation nor swimming performance are in themselves endpoints specific to TH disruption since they can be affected through various mechanisms. The strength of an AOP-based approach, however, lies in linking these adverse outcomes to an endocrine mechanism, providing biologically plausible support that the adversity is effectively the consequence of the endocrine mechanism. In the case of TH disruption in fish, the strong evidence for the relationship between reduced TH levels and impaired swim bladder inflation is crucial in this respect [18, 21]. Measurements of altered TH levels thus increase the diagnostic value of general endpoints such as growth and swim bladder inflation by placing these endpoints in a TH disruption context based on the causal linkages in the AOP network. Specifically, the combination of whole-body TH measurements (T4, T3) and the assessment of swim bladder inflation (both chambers) and swimming performance could be included as an AOP-based suite of endpoints in any test guideline using zebrafish or fathead minnow early-life stages. This includes the FELS test, FSDT, ZEOGRT and FLCT. In the European Union, the FELS test is

279 the most important standard ecotoxicological data requirement for industrial chemicals (REACH), and
280 active substances in biocides (EU 528/2012 [46]) and plant protection products (EU 1107/2009 [47]).
281 Increasing the diagnostic value of the FELS test for the detection of TH disrupters may therefore
282 significantly increase the efficiency of chemical safety evaluation in terms of cost and use of animals.
283 Future development of new AOPs linking TH disruption to adverse effects that are already being
284 assessed as a part of these test guidelines (e.g., growth) may further improve the significance of these
285 endpoints.

286

287 ## 4. Considerations for the further development and application of the AOP network

288 ### 4.1. Expanding the domain of applicability

289 The TH disruption AOP network to which the assays discussed in this case example are aligned (Figure
290 2) is included in the OECD AOP development programme workplan as Project 1.35 (*The AOP on*
291 *thyroperoxidase and/or deiodinase inhibition leading to impaired swim bladder inflation in fish during*
292 *early-life stages*) and is mainly based on studies using zebrafish and fathead minnow. A first logical
293 step in expanding the applicability of the AOP network is to assess its relevance to other species that
294 are frequently used in existing fish test guidelines, such as the Japanese rice fish (also known as the
295 medaka), three-spined stickleback and rainbow trout. Second, since TH disruption can be caused
296 through many different mechanisms (e.g., iodine uptake inhibition, increased liver TH clearance), the
297 AOP network related to effects in fish could be expanded to include a larger variety of molecular
298 initiating events [41]. Further, several other endpoints and biomarkers that have been shown to respond
299 to impaired thyroid function and/or altered TH levels could be added, including gene expression, eye
300 development (e.g., size, pigmentation, retina histology), skin pigmentation, scale development,
301 impaired fin development, and thyroid histopathology assessing thyroid follicle morphology e.g., using
302 staining techniques or transgenic fish [30, 48, 49]. The development of AOPs covering these adverse effects
303 would facilitate the assessment of their specificity and sensitivity in the context of TH disruption. In
304 addition, linking these AOPs to the existing AOP network would help expand the life stage applicability

from early-life stages to juveniles and reproductively active, adult fish for a range of species. Such efforts would make the AOP network relevant to a number of additional fish test guidelines, including the fish short-term reproduction assay (OECD TG 229), the 21-day fish assay (OECD TG 230), the androgenised female stickleback screen (OECD GD 148), the juvenile medaka anti-androgen screening assay (draft OECD GD) and the rapid androgen disruption adverse outcome reporter assay (draft OECD TG) at Level 3 of the CF, and the medaka extended one-generation reproduction test (OECD TG 240) at Level 5.

Tiered testing strategies for the evaluation of TH disrupting properties are being developed in parallel for human and environmental health [3, 7]. Evaluation of the hazards and risks of chemicals derived from tests for human health effects and environmental effects are largely separate processes, and sharing of data is uncommon. Human toxicology and ecotoxicology have historically used different models, terminologies and interpretation approaches. The AOP framework facilitates the application of similar strategies for developing assays and using them in a unified weight of evidence analysis. This provides opportunities for effectively bridging the gap between these two disciplines [50-52]. A relatively large number of AOPs related to TH disruption is currently being developed in the AOP-Wiki [53], involving a variety of species and taxonomic groups. Two AOPs focusing on TH disruption (leading from thyroperoxidase and sodium iodine transporter inhibition to neural outcomes in rodents) have been endorsed and published by the OECD so far [54, 55]. Based on the fact that well-known targets along the HPT-axis are highly conserved among vertebrate classes [56, 57], a cross-species TH disruption AOP network covering mammals, fish and amphibians is emerging from these datasets (conceptually visualized in Figure 3) [15, 41]. The underlying AOPs and their interrelationships were recently described in detail to support the use of *in vitro* assays for the evaluation of TH disruption [41]. Further development and biological validation of this larger AOP network can form the basis of a harmonized, integrated approach to testing and assessment of TH disrupters addressing both human and environmental health. Finally, while we presently focus on TH disrupting activity and fish, tiered testing strategies for other modes of endocrine disruption can in principle also be informed by

emerging AOPs and AOP networks including adverse effects with complex and multifactorial origins [58].

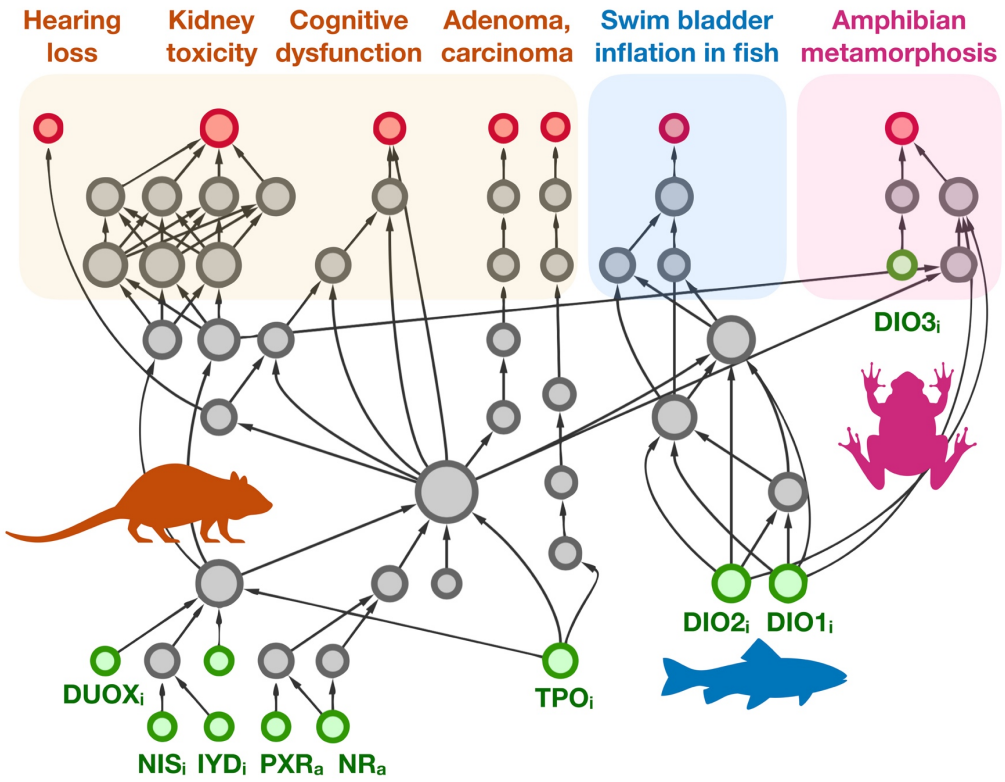


Figure 3. Graphical representation of a cross-species AOP network, present in the AOP-Wiki (date accessed: June 3, 2020), that links molecular initiating events (green circles) through impacts on circulating thyroid hormone levels, to adverse outcomes (red circles) in mammals, fish, and/or amphibians. Some of the AOPs in this AOP network are supported by a large amount of data that have been published in the peer-reviewed literature, while others are putative AOPs that are still under development. This figure therefore conceptually illustrates an AOP network approach across taxonomic groups but does not make any assumptions about the biological validity of the underlying AOPs. For that reason, the identity of intermediate key events has not been specified. AOPs, and hence the depicted AOP network, may be subject to change before they are formally finalized. DUOX: dual oxidase, NIS: sodium-iodide symporter, IYD: iodotyrosine deiodinase, PXR: pregnane X receptor, NR: hepatic nuclear receptor, TPO: thyroperoxidase, DIO (1,2,3): iodothyronine deiodinase (1,2,3), i: inhibition, a: activation. https://aopwiki.org/aops/xxx (xxx: AOP numbers 8, 42, 54, 119, 128, 134, 155, 156, 157, 158, 159, 162, 175, 176, 188, 189, 190, 191, 192, 193, 194).

347

348 4.2. Is the AOP network “Fit for Purpose”?

349 In the present paper we have illustrated how the AOP framework and associated data can be used to
350 address testing challenges in the context of the use of fish-based test guidelines to detect perturbation
351 of the thyroid hormone axis, an area of emphasis for multiple regulatory programs. A perceived
352 challenge to the use of AOPs and AOP networks for regulatory applications is that they are never
353 finished nor complete. Important additional molecular initiating events, key events, adverse effects,
354 and associated physiological processes, may not (yet) be captured by any given AOP (network). This
355 could potentially lead to a biased focus on specific pathways, mechanisms and processes, instead of
356 providing a broader and more holistic consideration of all of the available mechanistic evidence [59]. In
357 the case of our thyroid AOP network, important and well-known mechanisms affecting TH
358 concentrations (e.g., iodine uptake inhibition, increased liver TH clearance) are indeed not yet
359 included.

360 The critical question is at what point an AOP conveys knowledge that can help to support the use of
361 available, or readily produced, data for decision-making. To the extent that the answer will vary with
362 the nature of the decision, the question is: when is an AOP (network) fit for a particular purpose?
363 Although they are indeed so-called “living documents” that can evolve over time, AOPs are
364 nonetheless useful at different stages of completeness depending on the intended application. For
365 example, if the purpose of a regulatory activity/program is prioritization, less evidence and less
366 confidence may be required than if the purpose is quantitative risk assessment, where a higher level
367 of scientific confidence or even sophisticated quantitative understanding may be required [60]. Specific
368 criteria (e.g., the type and level of AOP complexity and confidence in the AOP, as well as external
369 review and assay validation) have recently been proposed to examine when AOPs, AOP networks, and
370 associated tools are fit for purpose in different regulatory and risk assessment contexts [61]. Ultimately,
371 although individual AOPs will often continue to be refined over time and associated AOP networks will

continue to broaden the toxicological space that can be addressed, even an initial AOP (network) can be useful in a decision-making process, providing that it demonstrably increases confidence in a decision [60].

Although knowledge and data gaps most certainly remain in our current thyroid AOP network, the current level of understanding is sufficient to support selection of assays for TH disruption, including those useful for assessing important *in vivo* apical effects in fish early-life stages [21]. Further expanding toxicological space (in terms of initiating events) can certainly increase the domain of applicability of this AOP network (see section 4.1). At the same time, there could be a concern that such an approach could lead to an increasingly large number of assays, potentially complicating and even slowing down risk assessments by introducing an ever-growing list of endpoints to evaluate. In most tiered testing approaches however, data do not necessarily need to be collected at all tiers. Data requirements depend on the availability and nature of existing information and the needs for testing and assessment, not the list of all potentially relevant information. The addition of TH disruption assays/endpoints to existing test guidelines significantly improves the capacity of these guidelines to evaluate TH disruption by providing an improved level of flexibility in selecting assays corresponding to specific information requirements, while at the same time reducing the need for additional testing. Both in the US and the EU it is becoming increasingly evident that existence of such "fit for purpose" AOPs accelerates the regulatory process through enabling use of alternative data sources such as computational, molecular, and *in vitro* tools.

Finally, an important consideration in the context of assessing endocrine-active chemicals, is the necessity of establishing a causal or plausible relationship between changes in endocrine signaling (a molecular initiating event) and an adverse outcome for supporting regulatory decision-making. The development of AOPs such as those in the thyroid AOP network explicitly addresses the biological plausibility of the link between an adverse effect and endocrine activity. As such, this directly addresses the scientific criteria for the identification of endocrine-disrupting chemicals as outlined in for example the EU biocidal products (2017/2100 [62]) and plant protection products (2018/605 [63])

398 regulations. Some legislative initiatives and risk assessment strategies, however, do not necessarily
399 require data at different levels of biological organization nor an explicit link between an adverse effect
400 and a specific endocrine disruption mechanism. In these instances, data documenting an adverse
401 endocrine specific effect, such as altered sex ratio, are sufficient for regulatory decision-making. This
402 approach is, however, not straightforward in the case of TH disruption since adverse outcomes are
403 often non-specific and could also be caused by other endocrine and non-endocrine mechanisms.
404 Hence, AOP-based approaches are helpful in such scenarios by placing the observed effects in a TH
405 disruption context based on the linkages in the AOP network.
406
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References

1. Matthiessen, P.; Ankley, G. T.; Biever, R. C.; Bjerregaard, P.; Borgert, C.; Brugger, K.; Blankinship, A.; Chambers, J.; Coady, K. K.; Constantine, L.; Dang, Z.; Denslow, N. D.; Dreier, D. A.; Dungey, S.; Gray, L. E.; Gross, M.; Guiney, P. D.; Hecker, M.; Holbech, H.; Iguchi, T.; Kadlec, S.; Karouna-Renier, N. K.; Katsiadaki, I.; Kawashima, Y.; Kloas, W.; Krueger, H.; Kumar, A.; Lagadic, L.; Leopold, A.; Levine, S. L.; Maack, G.; Marty, S.; Meador, J.; Mihaich, E.; Odum, J.; Ortego, L.; Parrott, J.; Pickford, D.; Roberts, M.; Schaefers, C.; Schwarz, T.; Solomon, K.; Verslycke, T.; Weltje, L.; Wheeler, J. R.; Williams, M.; Wolf, J. C.; Yamazaki, K., Recommended approaches to the scientific evaluation of ecotoxicological hazards and risks of endocrine-active substances. *Integr Environ Assess Manag* **2017,** *9999*, 1-13.
2. Crofton, K. M., Thyroid disrupting chemicals: mechanisms and mixtures. *International Journal of Andrology* **2008,** *31* (2), 209-222.
3. Murk, A. J.; Rijntjes, E.; Blaauboer, B. J.; Clewell, R.; Crofton, K. M.; Dingemans, M. M. L.; Furlow, J. D.; Kavlock, R.; Kohrle, J.; Opitz, R.; Traas, T.; Visser, T. J.; Xia, M. H.; Gutleb, A. C., Mechanism-based testing strategy using in vitro approaches for identification of thyroid hormone disrupting chemicals. *Toxicology in Vitro* **2013,** *27* (4), 1320-1346.
4. Kortenkamp, A.; Martin, O.; Baynes, A.; Silva, E.; Axelstad, M.; Hass, U., Supporting the organisation of a workshop on thyroid disruption – Final Report. In *Framework Contract ENV.A.3/FRA/2014/0029 on implementation of the Community strategy on Endocrine Disrupters*, Publications Office of the European Union: Luxembourg, 2017.
5. Bopp, S.; Nepelska, M.; Halder, M.; Munn, S., Expert survey on identification of gaps in available test methods for evaluation of endocrine disruptors. In *JRC Technical Reports*, European Union: Luxembourg, 2017.
6. EFSA, Scientific opinion on the hazard assessment of endocrine disruptors: Scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment. EFSA Journal: 2013; Vol. 11, p 3132.
7. OECD, Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. In *OECD Series on Testing and Assessment*, OECD Publishing: Paris, 2018.
8. WHO/UNEP, State of the science of endocrine disrupting chemicals - 2012. In *ISBN: 978 92 4 150503 1*, Bergman Å, H. J., Jobling S, Kidd KA, Zoeller RT, Ed. WHO and UNEP: Geneva (CH), 2013.
9. Coady, K. K.; Biever, R. C.; Denslow, N. D.; Gross, M.; Guiney, P. D.; Holbech, H.; Karouna-Renier, N. K.; Katsiadaki, I.; Krueger, H.; Levine, S. L.; Maack, G.; Williams, M.; Wolf, J. C.; Ankley, G. T., Current Limitations and Recommendations to Improve Testing for the Environmental Assessment of Endocrine Active Substances. *Integrated Environmental Assessment and Management* **2017,** *13* (2), 302-316.
10. Burden, N.; Benstead, R.; Clook, M.; Doyle, I.; Edwards, P.; Maynard, S. K.; Ryder, K.; Sheahan, D.; Whale, G.; van Egmond, R.; Wheeler, J. R.; Hutchinson, T. H., Advancing the 3Rs in regulatory ecotoxicology: A pragmatic cross-sector approach. *Integrated Environmental Assessment and Management* **2016,** *12* (3), 417-421.
11. OECD, OECD conceptual framework for testing and assessment of endocrine disruptors. OECD: Paris (FR), 2012.

12. Browne, P.; Noyes, P. D.; Casey, W. M.; Dix, D. J., Application of Adverse Outcome Pathways to US EPA's Endocrine Disruptor Screening Program. *Environmental Health Perspectives* **2017,** *125* (9).
13. Ankley, G. T.; Bennett, R. S.; Erickson, R. J.; Hoff, D. J.; Hornung, M. W.; Johnson, R. D.; Mount, D. R.; Nichols, J. W.; Russom, C. L.; Schmieder, P. K.; Serrrano, J. A.; Tietge, J. E.; Villeneuve, D. L., Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry* **2010,** *29* (3), 730-741.
14. Ankley, G.; Edwards, S., The adverse outcome pathway: A multifaceted framework supporting 21st century toxicology. *Current Opinion in Toxicology* **2018,** *9*, 1-7.
15. Knapen, D.; Angrish, M. M.; Fortin, M. C.; Katsiadaki, I.; Leonard, M.; Margiotta-Casaluci, L.; Munn, S.; O'Brien, J. M.; Pollesch, N.; Smith, L. C.; Zhang, X. W.; Villeneuve, D. L., Adverse outcome pathway networks I: Development and applications. *Environmental Toxicology and Chemistry* **2018,** *37* (6), 1723-1733.
16. Villeneuve, D.; Angrish, M.; Fortin, M.; Katsiadaki, I.; Leonard, M.; Margiotta-Casaluci, L.; Munn, S.; O'Brien, J.; Pollesch, N.; Smith, L.; Zhang, X.; Knapen, D., Adverse Outcome Pathway Networks II: Network Analytics. *Environ Toxicol Chem* **2018,** *doi: 10.1002/etc.4124*.
17. Hagenaars, A.; Stinckens, E.; Vergauwen, L.; Bervoets, L.; Knapen, D., PFOS affects posterior swim bladder chamber inflation and swimming performance of zebrafish larvae. *Aquatic Toxicology* **2014,** *157*, 225-235.
18. Stinckens, E.; Vergauwen, L.; Schroeder, A.; Maho, W.; Blackwell, B.; Witters, H.; Blust, R.; Ankley, G.; Covaci, A.; Villeneuve, D.; Knapen, D., Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part II: Zebrafish. *Aquatic Toxicology* **2016,** *173*, 204-217.
19. Nelson, K.; Schroeder, A.; Ankley, G.; Blackwell, B.; Blanksma, C.; Degitz, S.; Flynn, K.; Jensen, K.; Johnson, R.; Kahl, M.; Knapen, D.; Kosian, P.; Milsk, R.; Randolph, E.; Saari, T.; Stinckens, E.; Vergauwen, L.; Villeneuve, D., Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part I: Fathead minnow. *Aquatic Toxicology* **2016,** *173*, 192-203.
20. Vergauwen, L.; Cavallin, J. E.; Ankley, G. T.; Bars, C.; Gabriels, I. J.; Michiels, E. D. G.; Fitzpatrick, K. R.; Periz-Stanacev, J.; Randolph, E. C.; Robinson, S. L.; Saari, T. W.; Schroeder, A. L.; Stinckens, E.; Swintek, J.; Van Cruchten, S. J.; Verbueken, E.; Villeneuve, D. L.; Knapen, D., Gene transcription ontogeny of hypothalamic-pituitary-thyroid axis development in early-life stage fathead minnow and zebrafish. *General and Comparative Endocrinology* **2018,** *266*, 87-100.
21. Stinckens, E.; Vergauwen, L.; Blackwell, B. R.; Ankley, G. T.; Villeneuve, D. L.; Knapen, D., Effect of thyroperoxidase and deiodinase inhibition on anterior swim bladder inflation in the zebrafish. *Environmental Science & Technology* **2020,** *54*, 6213-6223.
22. Stinckens, E.; Vergauwen, L.; Ankley, G. T.; Blust, R.; Darras, V. M.; Villeneuve, D. L.; Witters, H.; Volz, D. C.; Knapen, D., An AOP-based alternative testing strategy to predict the impact of thyroid hormone disruption on swim bladder inflation in zebrafish. *Aquatic Toxicology* **2018,** *200*, 1-12.
23. Cavallin, J. E.; Ankley, G. T.; Blackwell, B. R.; Blanksma, C. A.; Fay, K. A.; Jensen, K. M.; Kahl, M. D.; Knapen, D.; Kosian, P. A.; Poole, S. T.; Randolph, E. C.; Schroeder, A. L.; Vergauwen, L.; Villeneuve, D. L., Impaired swim bladder inflation in early life stage fathead

minnows exposed to a deiodinase inhibitor, iopanoic acid. *Environmental Toxicology and Chemistry* **2017,** *36* (11), 2942-2952.
24. Villeneuve, D.; Volz, D. C.; Embry, M. R.; Ankley, G. T.; Belanger, S. E.; Leonard, M.; Schirmer, K.; Tanguay, R.; Truong, L.; Wehmas, L., Investigating alternatives to the fish early-life stage test: a strategy for discovering and annotating adverse outcome pathways for early fish development. *Environmental Toxicology and Chemistry* **2014,** *33* (1), 158-169.
25. Houbrechts, A. M.; Delarue, J.; Gabriels, I. J.; Sourbron, J.; Darras, V. M., Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. *Endocrinology* **2016,** *157* (9), 3668-3681.
26. Heijlen, M.; Houbrechts, A.; Bagci, E.; Van Herck, S.; Kersseboom, S.; Esguerra, C.; Blust, R.; Visser, T.; Knapen, D.; Darras, V., Knockdown of type 3 iodothyronine deiodinase severely perturbs both embryonic and early larval development in zebrafish. *Endocrinology* **2014,** *155*, 1547-1559.
27. Bagci, E.; Heijlen, M.; Vergauwen, L.; Hagenaars, A.; Houbrechts, A. M.; Esguerra, C. V.; Blust, R.; Darras, V. M.; Knapen, D., Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. *PLOS One* **2015,** *10*, e0123285.
28. Godfrey, A.; Hooser, B.; Abdelmoneim, A.; Horzmann, K. A.; Freemanc, J. L.; Sepulveda, M. S., Thyroid disrupting effects of halogenated and next generation chemicals on the swim bladder development of zebrafish. *Aquatic Toxicology* **2017,** *193*, 228-235.
29. Jomaa, B.; Hermsen, S. A. B.; Kessels, M. Y.; van den Berg, J. H. J.; Peijnenburg, A. A. C. M.; Aarts, J. M. M. J. G.; Piersma, A. H.; Rietjens, I. M. C. M., Developmental Toxicity of Thyroid-Active Compounds in a Zebrafish Embryotoxicity Test. *Altex-Alternatives to Animal Experimentation* **2014,** *31* (3), 303-317.
30. Walpita, C. N.; Crawford, A. D.; Janssens, E. D.; Van der Geyten, S.; Darras, V. M., Type 2 iodothyronine deiodinase is essential for thyroid hormone-dependent embryonic development and pigmentation in zebrafish. *Endocrinology* **2009,** *150* (1), 530-9.
31. Walpita, C. N.; Crawford, A. D.; Darras, V. M., Combined antisense knockdown of type 1 and type 2 iodothyronine deiodinases disrupts embryonic development in zebrafish (Danio rerio). *Gen Comp Endocrinol* **2010,** *166* (1), 134-41.
32. AOP-Wiki. https://aopwiki.org/aops/155-159.
33. Robertson, G. N.; McGee, C. A. S.; Dumbarton, T. C.; Croll, R. P.; Smith, F. M., Development of the swimbladder and its innervation in the zebrafish, Danio rerio. *Journal of Morphology* **2007,** *268* (11), 967-985.
34. Power, D. M.; Llewellyn, L.; Faustino, M.; Nowell, M. A.; Bjornsson, B. T.; Einarsdottir, I. E.; Canario, A. V.; Sweeney, G. E., Thyroid hormones in growth and development of fish. *Comp Biochem Physiol C Toxicol Pharmacol* **2001,** *130* (4), 447-59.
35. Villeneuve, D. L.; Crump, D.; Garcia-Reyero, N.; Hecker, M.; Hutchinson, T. H.; LaLone, C. A.; Landesmann, B.; Lettieri, T.; Munn, S.; Nepelska, M.; Ottinger, M. A.; Vergauwen, L.; Whelan, M., Adverse Outcome Pathway (AOP) Development I: Strategies and Principles. *Toxicological Sciences* **2014,** *142* (2), 312-320.
36. OECD, New scoping document on in vitro and ex vivo assays for the identification of modulators of thyroid hormone signalling. In *Series on Testing and Assessment No. 207*, OECD: Paris, 2014; Vol. ENV/JM/MONO(2014)23.
37. Paul Friedman, K.; Watt, E. D.; Hornung, M. W.; Hedge, J. M.; Judson, R. S.; Crofton, K. M.; Houck, K. A.; Simmons, S. O., Tiered High-Throughput Screening Approach to

559 Identify Thyroperoxidase Inhibitors Within the ToxCast Phase I and II Chemical Libraries.
560 *Toxicological Sciences* **2016,** *151* (1), 160-180.
561 38. Wang, J.; Hallinger, D. R.; Murr, A. S.; Buckalew, A. R.; Simmons, S. O.; Laws, S. C.;
562 Stoker, T. E., High-Throughput Screening and Quantitative Chemical Ranking for Sodium-
563 Iodide Symporter Inhibitors in ToxCast Phase I Chemical Library. *Environmental Science &*
564 *Technology* **2018,** *52* (9), 5417-5426.
565 39. Olker, J. H.; Korte, J. J.; Denny, J. S.; Hartig, P. C.; Cardon, M. C.; Knutsen, C. N.;
566 Kent, P. M.; Christensen, J. P.; Degitz, S. J.; Hornung, M. W., Screening the ToxCast Phase 1,
567 Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases.
568 *Toxicological Sciences* **2019,** *168* (2), 430-442.
569 40. Dong, H. Y.; Wade, M. G., Application of a nonradioactive assay for high throughput
570 screening for inhibition of thyroid hormone uptake via the transmembrane transporter
571 MCT8. *Toxicology in Vitro* **2017,** *40*, 234-242.
572 41. Noyes, P. D.; Friedman, K. P.; Browne, P.; Haselman, J. T.; Gilbert, M. E.; Hornung,
573 M. W.; Barone, S.; Crofton, K. M.; Laws, S. C.; Stoker, T. E.; Simmons, S. O.; Tietge, J. E.;
574 Degitz, S. J., Evaluating Chemicals for Thyroid Disruption: Opportunities and Challenges with
575 in Vitro Testing and Adverse Outcome Pathway Approaches. *Environmental Health*
576 *Perspectives* **2019,** *127* (9).
577 42. Hassan, I.; El-Masri, H.; Kosian, P. A.; Ford, J.; Degitz, S. J.; Gilbert, M. E.,
578 Neurodevelopment and Thyroid Hormone Synthesis Inhibition in the Rat: Quantitative
579 Understanding Within the Adverse Outcome Pathway Framework. *Toxicological Sciences*
580 **2017,** *160* (1), 57-73.
581 43. Thienpont, B.; Tingaud-Sequeira, A.; Prats, E.; Barata, C.; Babin, P. J.; Raldua, D.,
582 Zebrafish Eleutheroembryos Provide a Suitable Vertebrate Model for Screening Chemicals
583 that Impair Thyroid Hormone Synthesis. *Environmental Science & Technology* **2011,** *45* (17),
584 7525-7532.
585 44. EC, EU Parliament and Council Directive 2010/63/EU of 22 September 2010 on the
586 protection of animals used for scientific purposes. 2010.
587 45. Sobanska, M.; Scholz, S.; Nyman, A. M.; Cesnaitis, R.; Alonso, S. G.; Kluver, N.;
588 Kuhne, R.; Tyle, H.; de Knecht, J.; Dang, Z. C.; Lundbergh, I.; Carlon, C.; De Coen, W.,
589 Applicability of the fish embryo acute toxicity (FET) test (OECD 236) in the regulatory
590 context of Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH).
591 *Environmental Toxicology and Chemistry* **2018,** *37* (3), 657-670.
592 46. EC, Regulation (EU) No 528/2012 of the European Parliament and of the Council of
593 22 May 2012 concerning the making available on the market and use of biocidal products.
594 2012.
595 47. EC, Regulation (EC) No 1107/2009 of the European Parliament and of the Council of
596 21 October 2009 concerning the placing of plant protection products on the market and
597 repealing Council Directives 79/117/EEC and 91/414/EEC. 2009.
598 48. Baumann, L.; Ros, A.; Rehberger, K.; Neuhauss, S. C. F.; Segner, H., Thyroid
599 disruption in zebrafish (Danio rerio) larvae: Different molecular response patterns lead to
600 impaired eye development and visual functions. *Aquatic Toxicology* **2016,** *172*, 44-55.
601 49. van der Ven, L. T. M.; van den Brandhof, E. J.; Vos, J. H.; Power, D. M.; Wester, P.
602 W., Effects of the antithyroid agent propylthiouracil in a partial life cycle assay with
603 zebrafish. *Environmental Science & Technology* **2006,** *40* (1), 74-81.
604 50. Perkins, E. J.; Ankley, G. T.; Crofton, K. M.; Garcia-Reyero, N.; LaLone, C. A.;
605 Johnson, M. S.; Tietge, J. E.; Villeneuve, D. L., Current Perspectives on the Use of Alternative

606 Species in Human Health and Ecological Hazard Assessments. *Environmental Health*
607 *Perspectives* **2013,** *121* (9), 1002-1010.
608 51. Rivetti, C.; Allen, T. E. H.; Brown, J. B.; Butler, E.; Carmichael, P. L.; Colbourne, J. K.;
609 Dent, M.; Falciani, F.; Gunnarsson, L.; Gutsell, S.; Harrill, J. A.; Hodges, G.; Jennings, P.;
610 Judson, R.; Kienzler, A.; Margiotta-Casaluci, L.; Muller, I.; Owen, S. F.; Rendal, C.; Russell,
611 P. J.; Scott, S.; Sewell, F.; Shah, I.; Sorrel, I.; Viant, M. R.; Westmoreland, C.; White, A.;
612 Carnpos, B., Vision of a near future: Bridging the human health-environment divide. Toward
613 an integrated strategy to understand mechanisms across species for chemical safety
614 assessment. *Toxicology in Vitro* **2020,** *62*.
615 52. McArdle, M.; Freeman, E. L.; Staveley, J.; Ortego, L.; Coady, K.; Weltje, L.; Weyers,
616 A.; Wheeler, J. R.; Bone, A. J., Critical Review of Read-Across Potential in Testing for
617 Endocrine-Related Effects in Vertebrate Ecological Receptors. *Environmental Toxicology and*
618 *Chemistry* **2020**.
619 53. AOP-Wiki www.aopwiki.org.
620 54. Rolaki, A.; Pistollato, F.; Munn, S.; Bal-Price, A., Adverse Outcome Pathway on
621 Inhibition of Na+/I-symporter (NIS) leads to learning and memory impairment. In *OECD*
622 *Series on Adverse Outcome Pathways No. 14*, OECD Publishing: Paris, 2019.
623 55. Crofton, K.; Gilbert, M.; Paul Friedman, K.; Demeneix, B.; Marty, M.; Zoeller, T.,
624 Adverse Outcome Pathway on inhibition of Thyroperoxidase and subsequent adverse
625 neurodevelopmental outcomes in mammals. In *OECD Series on Adverse Outcome Pathways*
626 *No. 13*, OECD Publishing: Paris, 2019.
627 56. LaLone, C.; Villeneuve, D. L.; Doering, J. A.; Blackwell, B. R.; Transue, T. R.;
628 Simmons, C. W.; Swintek, J.; Degitz, S.; Williams, A. J.; Ankley, G. T., Evidence for Cross
629 Species Extrapolation of Mammalian-Based High-Throughput Screening Assay Results.
630 *Environmental Science & Technology* **2018**.
631 57. Sachs, L. M.; Buchholz, D. R., Frogs model man: In vivo thyroid hormone signaling
632 during development. *Genesis* **2017,** *55* (1-2), 10.
633 58. Knapen, D.; Vergauwen, L.; Villeneuve, D. L.; Ankley, G. T., The potential of AOP
634 networks for reproductive and developmental toxicity assay development. *Reproductive*
635 *Toxicology* **2015,** *56*, 52-55.
636 59. La Merrill, M. A.; Vandenberg, L. N.; Smith, M. T.; Goodson, W.; Browne, P.;
637 Patisaul, H. B.; Guyton, K. Z.; Kortenkamp, A.; Cogliano, V. J.; Woodruff, T. J.; Rieswijk, L.;
638 Sone, H.; Korach, K. S.; Gore, A. C.; Zeise, L.; Zoeller, R. T., Consensus on the key
639 characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nature*
640 *Reviews Endocrinology* **2020,** *16* (1), 45-57.
641 60. Perkins, E. J.; Antczak, P.; Burgoon, L.; Falciani, F.; Garcia-Reyero, N.; Gutsell, S.;
642 Hodges, G.; Kienzler, A.; Knapen, D.; McBride, M.; Willett, C., Adverse Outcome Pathways
643 for Regulatory Applications: Examination of Four Case Studies With Different Degrees of
644 Completeness and Scientific Confidence. *Toxicological Sciences* **2015,** *148* (1), 14-25.
645 61. Coady, K.; Browne, P.; Embry, M.; Hill, T.; Leinala, E.; Steeger, T.; Maslankiewicz,
646 L.; Hutchinson, T., When Are Adverse Outcome Pathways and Associated Assays "Fit for
647 Purpose" for Regulatory Decision-Making and Management of Chemicals? *Integrated*
648 *Environmental Assessment and Management* **2019,** *15* (4), 633-647.
649 62. EC, Commission delegated regulation (EU) 2017/2100 of 4 September 2017 setting
650 out scientific criteria for the determination of endocrine-disrupting properties pursuant to
651 Regulation (EU) No 528/2012 of the European Parliament and Council. 2017.

63. EC, Commission Regulation (EU) 2018/605 of 19 April 2018 amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties. 2018.